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14. ABSTRACT

The purpose of this DOD PRTA is to investigate the role of epithelial plasticity in the promotion of metastasis in advanced prostate cancer through a sequential interrogation of biomarkers in primary prostate tumors, metastases, and circulating tumor cells (CTCs). In this 2011-2012 annual report, we provide updates on our investigation of EMT biology in metastatic prostate cancer, with particular emphasis on CTC biology as a biomarker of disease progression and metastasis. We also describe our ongoing investigation into novel CTC phenotypes in men with metastatic castration-resistant prostate cancer (CRPC), localized PC, and the investigation of CTCs for DNA biomarkers (copy number variatiations) that may each shed light on the molecular pathophysiology of metastatic spread. We provide evidence for the common co-expression of epithelial and mesenchymal/EMT biomarkers in CTCs from patients with metastatic castration-resistant prostate cancer (CRPC) as well as the common expression of stem cell biomarkers in these CTCs. This data provides strong evidence for the importance of EMT to prostate cancer metastasis in humans. Based on these results, we have developed a novel CTC capture method based on this EMT biology to identify non-epithelial OB-cadherin positive cells/CTCs which are previously underdetected using conventional CTC assays; further molecular characterization of these cellular events is ongoing. Together these aims will provide insight into metastasis biology in PC and lead to the identification of relevant therapeutic targets directed against this lethal metastatic process.

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INTRODUCTION

In 2012, over 80 U.S. men will die every day from prostate cancer (PC)[1]. Many deaths could potentially be prevented through identification and treatment directed at high risk disease. Currently, clinical/pathologic measures (i.e. PSA, stage, grade) provide no biologic insights into the process by which PC cells metastasize and become lethal. The measurement of circulating tumor cells (CTCs) in men with PC represents one biomarker with prognostic and predictive implications[2]. Many patients with metastatic PC, however, have undetectable CTCs, limiting clinical utility. We have identified epithelial-mesenchymal transitions (EMT) in experimental models of PC in which the cellular phenotype undergoes reversible (plastic) changes from an epithelial to a mesenchymal nature facilitating metastatic spread, followed by epithelial reversion in the target metastatic organ[3]. While in the active process of metastasis, CTCs may possess a mesenchymal/plastic phenotype, and thus may not be captured by existing epithelial-based CTC technologies. In this PRTA 2011-2012 annual report, we provide updates on our investigation of EMT biology in metastatic prostate cancer, with particular emphasis on circulating tumor cell biology as a biomarker of disease progression and metastasis. We also describe our ongoing investigation into novel CTC phenotypes in men with metastatic castration-resistant prostate cancer (CRPC), localized PC, and the investigation of CTCs for DNA biomarkers (copy number variations) that may each shed light on the molecular pathophysiology of metastatic spread. We provide evidence for the common co-expression of epithelial and mesenchymal/EMT biomarkers in CTCs from patients with metastatic castration-resistant prostate cancer (CRPC) as well as the common expression of stem cell biomarkers in these CTCs[4]. This data provides strong evidence for the importance of EMT to prostate cancer metastasis in humans. Based on these results, we have developed a novel CTC capture method based on this EMT biology to identify non-epithelial OB-cadherin positive cells/CTCs which are previously under detected using conventional CTC assays; further molecular characterization of these cellular events is ongoing. Together these aims will provide insight into metastasis biology in PC and lead to the identification of relevant targets for therapies directed against this lethal metastatic process.

BODY

Task 1: To link evidence of epithelial plasticity with adverse clinical features and PSA-based outcomes using a Durham VA Hospital prostate cancer tissue microarray and the SEARCH database (Months 1-36)

Key Research Accomplishments: See the year 1 annual report for a description of our work in optimizing this tissue microarray using the Ki-67 control antigen, which we found did correlate with PSA outcomes and high risk disease among men with localized prostate cancer who were undergoing radical prostatectomy. This data provided evidence as to the reliability of this TMA for biomarker studies using a known positive control biomarker. In this TMA, 207 men contributed RP tissues with linkage to clinical and pathologic data as well as outcomes data over a 4-5 year follow-up period. We link clinical data through the VA SEARCH database as described in specific aim 1 of this PRTA. In order to now assess EMT biomarkers in prostate tissue, we have selected 3 EMT biomarkers of interest, given their prior correlation with outcomes in other RP outcomes datasets: E-cadherin, vimentin, and N-cadherin[5;6].

For vimentin, a mouse anti-human and validated monoclonal antibody (DAKO IgG1, Clone V9) was used. For N-cadherin, a validated monoclonal mouse anti-human IgG1 (DAKO, IgG1, Clone 6G11) was used. And for E-cadherin, an IgG2a mouse monoclonal anti-human antibody (DAKO iGG1, clone NCH-38) was used. Control epithelial and stromal tissue was used to establish positive and negative conditions prior to the interpretation of staining expression by trained GU pathologists who were blinded to cancer outcome or patient identifiers. Scoring was performed only for epithelial elements rather than stromal. For vimentin and E-cadherin, scoring was 0 (absent), 1 (mild/focal expression), 2 (diffuse/intense expression). For N-cadherin, scoring was present or absent (0, 1).

We found that E-cadherin was uniformly expressed in localized prostate cancer with 85% having uniform, intense staining, and only 13% of TMA samples showing weaker expression; only 1.4% of samples lacked E-cadherin in epithelial cells as denoted by H&E staining. See **figure 1** for typical images of high-expression patterns of each of these proteins.

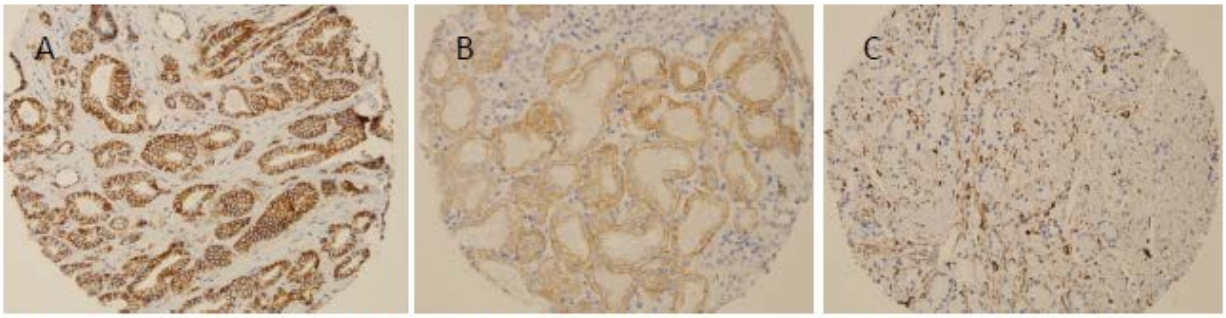


Figure 1. Prostate cancer TMA immunohistochemical staining examples from the Durham VA SEARCH database, stained for A) E-cadherin (2+), B) N-cadherin (1+), and C) vimentin (2+).

Given this strong uniform expression of E-cadherin, we found no correlation of high E-cadherin expression with Gleason sum at surgery. Rather, we found that higher risk tumors had higher E-cadherin expression (low E-cadherin expression seen in 28%, 11%, and 4% of Gleason 6, 7, and 8-10 tumors, respectively, p=0.023, see **figure 2**). High risk NCCN prostate cancers (PSA>20, T3 or higher, Gleason 8-10) were noted to uniformly have high E-cadherin expression (92% high, 0 absent), while NCCN low risk prostate cancers typically also have high expression (83% high, 2-3% absent). These findings suggest that in localized disease, both low and high grade tumors exhibit a predominantly epithelial phenotype with high E-cadherin expression. E-cadherin expression alone did not correlate with biochemical (PSA) recurrence after surgery and its loss of expression was not able to discriminate among men with or without recurrence (chi^2 p=0.68).

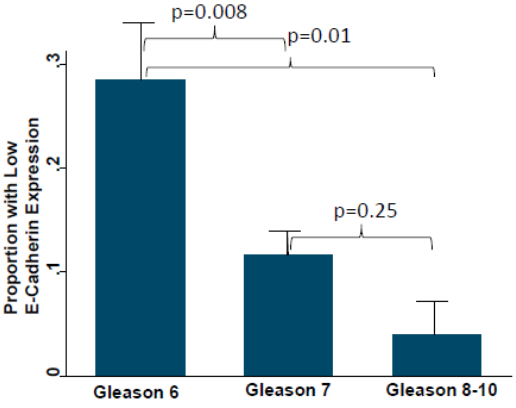


Figure 2. Low E-Cadherin expression according to post-operative Gleason sum

For vimentin, we found no association between expression and Gleason sum nor NCCN risk, nor with PSA outcomes after surgery. For example, high vimentin expression was seen in 33% of low risk NCCN tumors, 34% of intermediate risk, and 13.5% of high risk cancers (chi^2 p=0.058), and rather there was a trend for less vimentin expression in the primary for high risk adenocarcinomas. Likewise, Gleason 8-10 tumors overexpressed vimentin in 20.8% of cases, while low risk men, this was noted in 33% of cases (chi^2 p=0.56). High vimentin expression did not correlate with PSA recurrence (HR 0.73, p=0.22) in univariate analysis, while NCCN risk did (HR 2.1, p<0.0001). See **figure 3**.

For N-cadherin, we noted very little overexpression of this marker, despite its expression in stroma. In scored epithelial tumor cells for example, we only found overexpression of N-

cadherin in only 8/202 men. Thus, there is quite limited power to be able to demonstrate any associations with outcome, even if present, given the rarity of N-cadherin expression in this TMA. N-cadherin + tumors, however, were not more likely to be high grade, as 2.4%, 4.4%, and 4.4% of Gleason 6, 7, and 8-10 tumors expressed N-cadherin (chi^2=0.84). Likewise, we found no association with NCCN risk group (p=0.37), although numerically, higher NCCN risk men had higher levels of N-cadherin expression as compared to low risk NCCN men (5.4% high risk, 4.8% intermediate risk, 1.3% low risk). No association with PSA recurrence was noted for N-cadherin overexpression (HR 0.66, p=0.49). Interestingly, all of the N-cadherin over-expressing tumors also had high E-cadherin expression. We did not identify any men with both low E-cadherin expression and high N-cadherin expression. Finally, we found no correlation between vimentin overexpression and N-cadherin overexpression (p=0.84): 3-4% of men with low and high vimentin expression in their tumors had high N-cadherin. Thus, we were not able to confirm the findings of Gravdal et al[5;6] suggesting a relationship between vimentin or E to N-cadherin switching in the prostate with recurrence or adverse pathologic features.

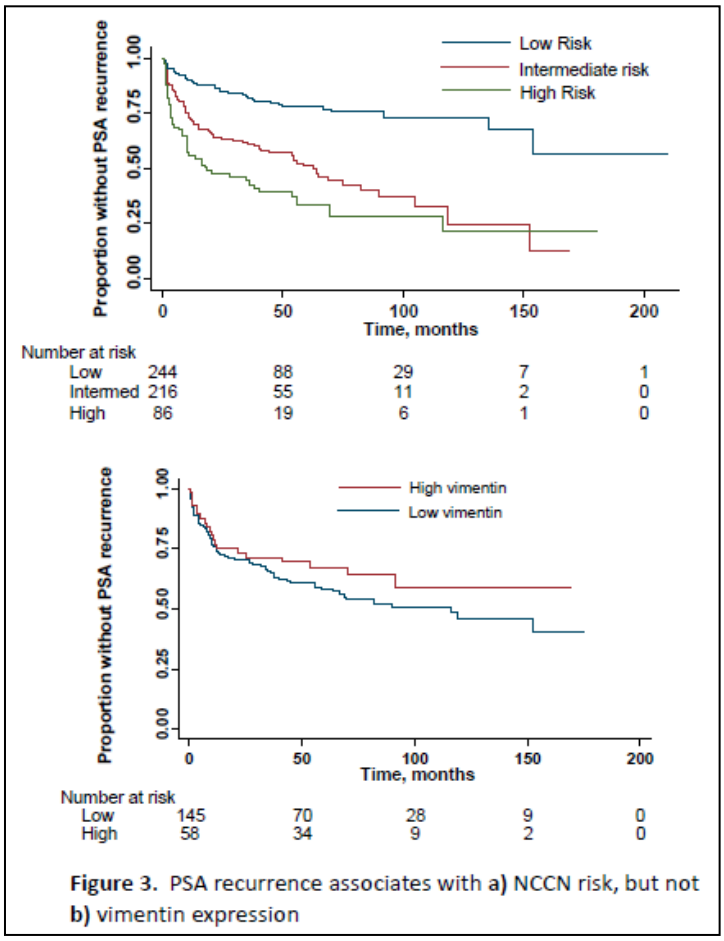


Figure 3. PSA recurrence associates with **a)** NCCN risk, but not **b)** vimentin expression

Several explanations may exist for this lack of association of vimentin and E/N cadherin expression with outcomes. The first is the limited number of men with high risk disease (only 37/204 men in this dataset with TMA data from SEARCH). While NCCN risk group in this men with TMA biomarker data did correlate with outcome (HR1.6 p=0.001), there is limited power for the analyses of these biomarkers for PSA recurrence. Likewise, biochemical recurrence may be a poor surrogate for metastatic disease and death, given the ability to salvage some men with post-operative radiation or hormonal therapy. In this dataset, only 3 men have developed metastatic prostate cancer or died of prostate cancer, thus leaving limited power to detect an association of these biomarkers with these more definitive outcomes. Over the coming 1-2 years, we will update this SEARCH database with 6-7 year prostate specific outcomes which may provide additional power to identify these associations. In addition, we are pursuing other clinically annotated TMA datasets for these EMT biomarker-outcome correlations, such as the DoD-funded North Carolina-Louisiana Prostate Cancer Project (386 subjects with TMA data and >4 year outcomes). Additional funding would be required for this larger-scope proposal. The lack of association between E/N-cadherin or vimentin expression in our TMA-SEARCH database may also reflect that EMT proteins may only be unregulated in the periphery or invasive front of tumors [7].

We have found these markers to be over-expressed in the circulating tumor cells from men with mCRPC and women with metastatic breast cancer (see below), and others have found these proteins to be expressed on CTCs in other solid tumors (reviewed in [8]). Thus, it is possible that standard pathologic examination of primary tumors may not be able to reveal overexpression of EMT biomarker, as the majority of the tumor itself bears an epithelial

phenotype. In addition, metastatic disease typically has an epithelial phenotype [9], and thus suggests that during invasion/metastasis, cancer cells undergo an EMT during initial invasion/intravasation into the circulation, then exist as transitional cells with an intermediate epithelial-mesenchymal phenotype during circulation, and then undergo an MET (mesenchymal-epithelial transition) during extravasation and metastatic colonization. This is supported by recent preclinical modeling experiments, suggesting epithelial plasticity and stemness drives this switching and metastasis[10]. Thus, an explanation for our overall findings in primary tumors and CTCs supports this plasticity biology, in which the visualization of these biomarkers in the primary and metastatic tumors (epithelial>mesenchymal burden) may be different than the actively invading and circulating cancer cells (both epithelial and mesenchymal features).

In years 3-5, in addition to updating our long term outcomes in the SEARCH database to increase the power of detecting associations with metastases, PSA recurrence, and death, we will ascertain the relationship of additional biomarkers with these outcomes. We have already developed and validated several EMT markers that will then be applied in the following order to the TMA in order to study the association of marker expression with PSA recurrence and other clinical/pathologic variables: 1) SNAIL (SNAI-1), 2) ZEB-1 and ZEB-2, 3) O-Cadherin, 4) CD133, 5) TWIST, and 6) FGFR2 IIIb isoform. We have spent the past year developing a polyclonal rabbit IgG with specificity against FGFR2 IIIb, given the link of this receptor isoform with EMT/MET[3;11]. This antibody is able to recognize the extracellular domain of the IIIb epithelial variant with no cross-reactivity against the IIIc mesenchymal isoform. Given the association of epithelial states with FGFR2 vIIIb expression and mesenchymal states with FGFR2 vIIIc expression, this is of great interest to our lab and as an EMT/MET biomarker. Further optimization of this antibody for IHC and IF applications in year 3 is planned.

Task 2: To identify the presence of mesenchymal and stem cell markers on circulating prostate cancer cells (CTCs) derived from men with castration-resistant prostate cancer and associated with adverse clinical outcomes (Months 1-18).

Key Research Accomplishments: We have successfully conducted this task, leading to the attached published manuscript (**Armstrong et al, Mol Cancer Res 2011[4]**) describing in detail our findings of EMT and stem cell markers in the majority of CTCs from men with metastatic castration-resistant prostate cancer and women with metastatic breast cancer (see last year's annual report). This data provided among the strongest and earliest evidence to date for the existence of EMT (epithelial-mesenchymal transition) in human cancer, particularly in circulating tumor cells. Given the importance of EMT to cancer metastasis and the lethal phenotype, as well as to the generation of stemness and chemoresistance, we believe these findings have profound implications for cancer biology. They suggest that EMT plays a strong role in prostate cancer metastasis during castration-resistant progression. The findings also suggest that our existing CTC assays may underdetect circulating tumor cells with more mesenchymal phenotypes, given that they are likely to have reduced or absent EpCAM expression. Please see manuscript for a detailed discussion of these implications and results.

In year 2, we have now taken this process to the next stage, through the capture and identification/characterization of N- and OB-cadherin expression CTCs in men with metastatic CRPC. In order to accomplish this task we developed a collaborative research agreement with Veridex/Janssen Pharmaceuticals, which provided the research equipment and some supplies for the development of a novel ferrofluidic antibody against these EMT proteins. We conducted a healthy volunteer study as well as a metastatic CRPC correlative study, with preliminary results defined in task 3 below. However, in order to define a "mesenchymal CTC", we needed to define a characterization protein that met the following criteria: 1) absent in control leukocytes and healthy volunteer peripheral blood mononuclear cells (PMBCs); 2) present in CTCs from patients with metastatic CRPC; 3) present in control cell lines with BOTH epithelial AND mesenchymal phenotypes. Our first protein to evaluate, vimentin, failed these criteria, as it was found to be expressed frequently in patient leukocytes, particularly during adhesion to glass

slides. Thus, we next characterized total beta-catenin expression, given its role in facilitating signaling from the cadherin family of adhesion molecules into the cytoplasm. We found that control leukocytes lacks beta-catenin, and that beta-catenin expression was present in both epithelial cell lines (LnCAP prostate, T47D breast) and mesenchymal cell lines (BT549 breast, PC-3 prostate). We also characterized the expression of beta-catenin in EpCAM captured CTCs from men with mCRPC through the Cellsearch method. Similar to our published work in (4), we characterized beta-catenin expression on a per cell basis and per-patient basis. In 5 men with progressive mCRPC who were about to start a new systemic regimen, we identified beta-catenin expression in the majority of CTCs identified (CK+ CD45- DAPI+ cells). Specifically, 100% of all men had at least 1 CTC that co-expressed beta-catenin, and 97/166 individual cells (mean 58%, range 44%-88%) co-expressed beta-catenin. While this was not as high as we reported for other EMT antigens, its lack of expression in leukocytes and expression during EMT and acquisition of mesenchymal properties led us to select this as a potential characterization protein for mesenchymal-like CTCs. Examples are shown in figure 4.

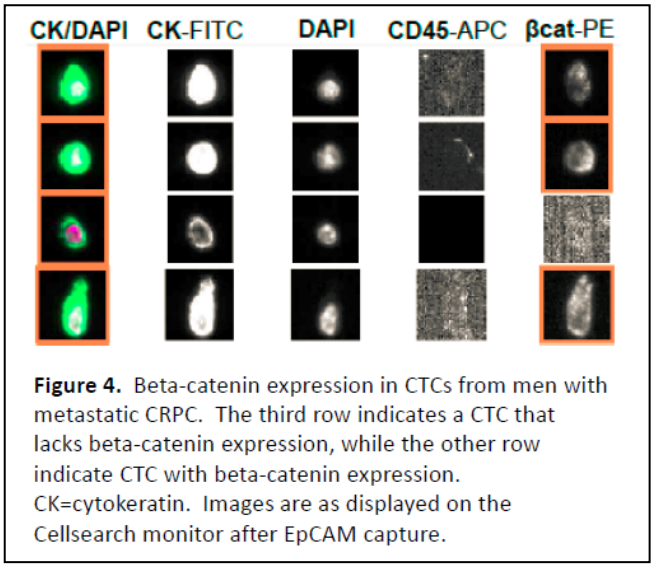


Figure 4. Beta-catenin expression in CTCs from men with metastatic CRPC. The third row indicates a CTC that lacks beta-catenin expression, while the other row indicate CTC with beta-catenin expression. CK=cytokeratin. Images are as displayed on the Cellsearch monitor after EpCAM capture.

We continue this task into year 3 as we examine additional biomarkers of EMT and stemness in CTCs beyond those initially examined, including aldehyde dehydrogenase (ALDH), FGFR2 isotypes, SNAIL, ZEB-1, and TWIST. Should we identify an EMT protein with a high prevalence of detection in both prostate and breast cancer CTCs as compared to beta-catenin, we may select this going forward for CTC characterization after capture.

Task 3: To refine the circulating tumor cell detection technology to capture circulating prostate tumor cells based on a mesenchymal surface marker rather than an epithelial marker (Months 37-60). This corresponds to pilot study 2 of SA2.

Key Research Accomplishments: Based on our above findings and patent (see last year's annual report), we have developed a collaborative research relationship with Veridex/Johnson and Johnson (now Janssen Pharmaceuticals) to develop a second generation CTC detection/capture assay based on task 2 above and the attached manuscript. These efforts are supported by the DOD PRTA (salary support). We have acquired a novel CTC detection instrument with a novel ferrofluid targeted at both N- and OB-cadherin rather than EpCAM. This work has led to the development of a **patent** focused on the novel detection of circulating tumor cells using an EMT antigen-based ferromagnetic capture method (submitted 9/24/10, international application number PCT/US10/50223).

Preliminary results from this work involving both healthy volunteers and men with mCRPC was presented at ASCO 2012 (abstract 10533, see attached). Patients with progressive, metastatic CRPC are enrolled prior to initiating a new systemic therapy. In this first phase, we enrolled 10 men for this pilot assessment of both N- and OB-cadherin CTC capture. Baseline characteristics are shown in table 1 below.

Table 1. Baseline characteristics of CRPC patients	**Results (n=10)**
Median age, y (range)	68 (57-74)
Race	
Caucasian, n (%)	7 (70)
Black, n (%)	3 (30)
Karnofsky performance status, median (range)	90 (80-100)
Gleason score, median (range)	8 (7-10)
Pain score >4, n (%)	4 (40)
Initial local therapy	
Prostatectomy, n (%)	3 (30)
External beam radiation, n (%)	3 (30)
None, n (%)	4 (40)
Laboratory values	
PSA ng/mL, median (range)	408 (7-4377)
LDH U/L, median (range)	220 (206-291)
Hemoglobin g/dL, median (range)	9.8 (8.8-12.1)
Alkaline phosphate U/L, median (range)	197 (57-463)
CTC count, median (range)	34 (1-1000)
Sites of metastasis	
Bone, n (%)	10 (100)
Liver, n (%)	2 (20)
Lung, n (%)	4 (40)
Lymph nodes only	0
Prior therapies	
Number of hormonal therapies, median (range)	4 (1-5)
Abiraterone, MDV3100, or TAK700, n (%)	7 (70)
Sipuleucel-T, n (%)	3 (30)
Docetaxel, n (%)	8 (80)
Cabazitaxel, n (%)	2 (20)
>1 chemotherapy, n (%)	2 (20)
Bone targeted therapy, n (%)	9 (90)
Palliative radiation, n (%)	3 (30)
Type of progression prior to study enrollment	
Imaging	8 (80)
Clinical (symptoms, PSA increase)	2 (20)

The men in this pilot study were quite treatment refractory, with over 80% having prior docetaxel for mCRPC, and many having progressed despite abiraterone or MDV3100 (70%), with 60% of men having visceral metastatic disease and 100% of men having bone metastatic disease; 40% of men were requiring narcotic analgesia for cancer pain.

In this pilot study, one CellSave® and two EDTA 10 mL tubes of blood are collected at baseline, at treatment cycle 3, and at progression. Using the CellSearch® system (Veridex, USA), CTCs are captured with anti-N-cadherin (N-cad) (Santa Cruz D4) or anti-O-cadherin (O-cad) (R&D Systems 283416) ferrofluid, then permeabilized and stained for further characterization. Mesenchymal CTCs (events) are defined as **β-catenin+, CD45-, nucleated intact cells**, based on the importance of β-catenin in EMT and on our preliminary data that β-catenin is absent in leukocytes. Performance was evaluated in healthy volunteers, control cells (PC3, BT549, osteoblasts), and in a pilot study of patients with metastatic CRPC. Mesenchymal CTC enumeration was compared with the standard EpCAM-based capture method. These results can be seen in the figure below (**Figure 5**). As can be seen with these distributional dot plots, in which each detected cellular event is plotted, we did not detect cellular N-cadherin + DAPI+ beta-catenin + CD45- events in healthy volunteers (mean 0.3 events, median 0). For OB (osteoblast)-cadherin, also known as CDH-11, we found greater heterogeneity in healthy volunteers, with a mean of 4.4 events (range 0-51, median 0). Thirty healthy volunteers contributed data to each of these plots for each novel CTC capture methods in order to provide a control detection threshold (negative control) for cellular events meeting our pre-specified novel CTC definition. Finally, given that some events were detected (see **figure 6 below**) that appeared spindle-shaped/elongated and cellular, we reasoned that these OB-cadherin positive events could be benign circulating cells, possibly of either endothelial or of bone marrow derived

origins. CD31 circulating endothelial cells (CECs) are rarely detectable in healthy volunteers, and CD31 is an endothelial cell protein[12]. When we co-stained by immunofluorescence our cellular events, we found that 100% of these events in healthy volunteers also expressed CD31, indicating their likely endothelial cell origin. When we use a further definition of beta-catenin + CD45 – DAPI + intact cell, healthy volunteers do not have cellular events detected by OB- or N-cadherin capture.

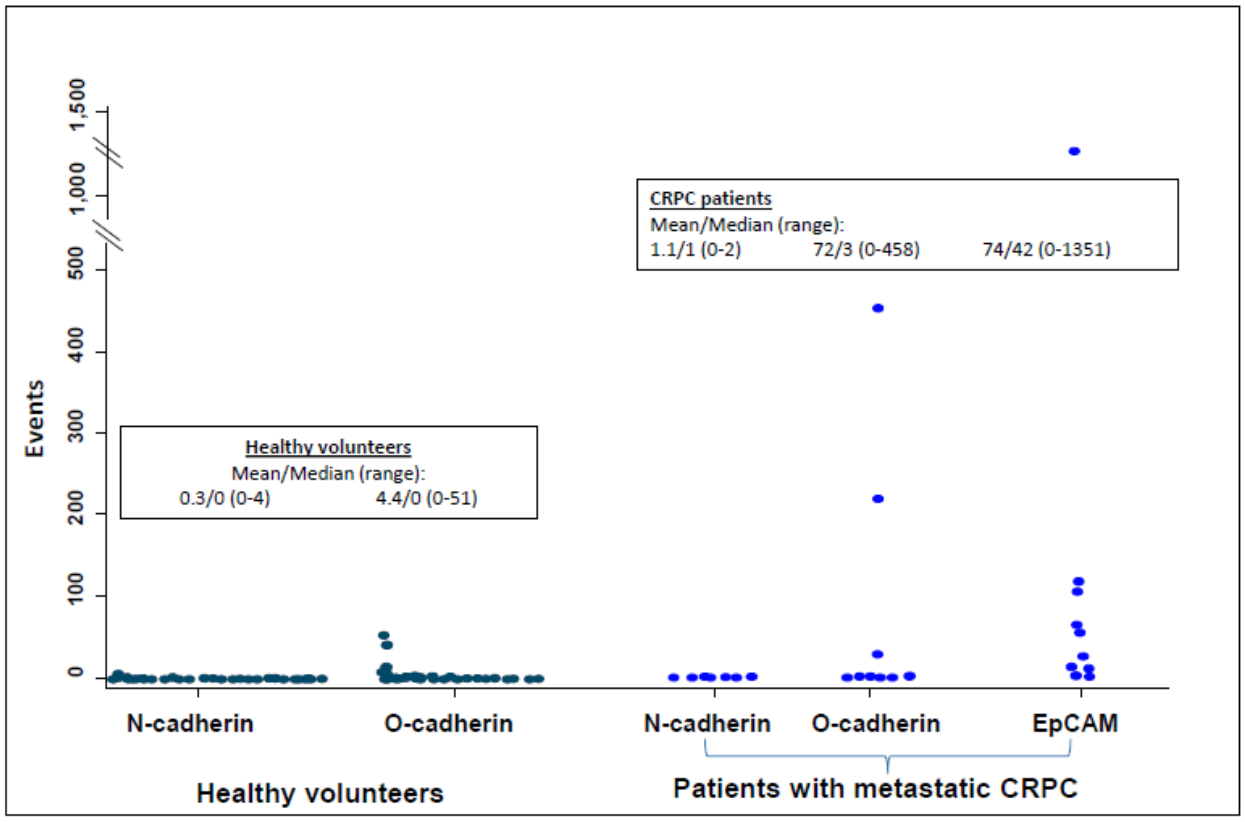
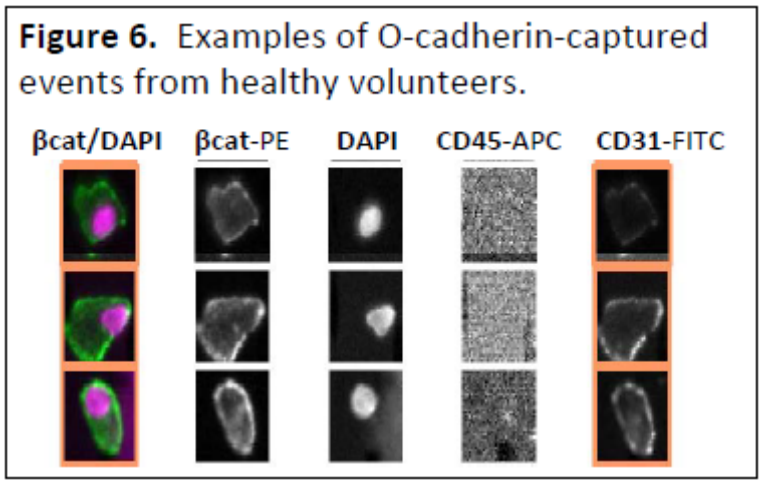


Figure 5. Events captured from healthy volunteers and CRPC patients using each ferrofluid



We next turned to positive control cell lines spiked into whole blood derived from healthy volunteers. Cells were spiked into EDTA containing whole blood and processed within 24 hours using the novel ferrofluids of interest. We used the T47D epithelial cell line for EpCAM control detection, and the PC-3 line for both N- and OB-cadherin expression, based on our previous data[4] that this cell lines heterogeneously expresses these markers in 30-50% of individual cells. We were able to detect both N- and OB-cadherin positive cells spiked into healthy volunteer blood ex vivo and processed using the novel antibody ferrofluid against N- and OB-

cadherin, and analyzed using the Cellsearch platform. A modified definition of a CTC was developed to exclude CD45+ and DAPI negative events and to only count beta-catenin + cellular events. The yield from spiking experiments of 500 cells ranged from 4-100% depending on the cell line used and antibody used for capture as shown in **table 2**.

Our positive control cells for N-cadherin (PC3 and PC3-9) demonstrated a percent recovery of 19-30%, consistent with our immunofluorescent (IF) data as these cells lines are highly heterogeneous. Our positive control cells for OB-cadherin (PC-3 and human osteoblasts) demonstrated a percent recovery of 22-28%, again consistent with great heterogeneity as seen on IF. We expected a greater percent recovery, however, for osteoblasts, which uniformly express OB-cadherin, and it is possible that our capture antibody is less efficient than the EpCAM ferrofluid. For positive control cells expressing EpCAM (T47D), our percent recovery was ~100% using the standard EpCAM ferrofluid. Potential explanations for the low rate of recovery for the N-cadherin and OB-cadherin ferrofluids include: 1) sensitivity to degradation over time (i.e. sensitivity to preservatives used, stability in EDTA); 2) differences in antigen expression in positive control cell lines; 3) differential avidity of the antibody for the antigen; 4) variability of intracellular vs. extracellular expression of these cadherin family members as compared to EpCAM; 5) differential binding of epitopes using IF vs. flow cytometry. Ongoing work in years 3-5 will be focused on each of these potential issues in order to optimize the percent recovery of positive control cells. One method we have developed to troubleshoot this issue has been to overexpress N-cadherin via plasmid transfection in PC-3 cells and then spiking these cells, which are also engineered to express Green Fluorescent Protein (GFP) for easy identification. The percent yield using the N-cadherin ferrofluid vs. flow sorting using GFP expression will be compared. Finally, an array of other antibodies against each antigen will be explored for optimal stability and conjugation to the ferromagnetic conjugate. Different preservatives (Cellsave, EDTA, others) will be examined to develop optimal conditions for measurement, and repeatability experiments over time will help to assess the reliability of these measures.

Percentage of cells recovered

	PC3	PC39	Osteoblasts	BT549	T47D
O-cadherin	28%	7%	22%	4%	4%
mean (range)	141 (0-352)	36 (34-39)	108 (13-207)	21	20
	n=6 (2 in dup)	n=1 (in trip)	n=3	n=1	n=1
N-cadherin	19%	30%	15%	27%	20%
mean (range)	95 (21-280)	151 (47-304)	73 (0-175)	133 (18-327)	103
	n=5 (1 in dup)	n=4 (2 in dup)	n=3	n=3 (2 in dup)	n=1

Table 2. Spiking studies of positive control cells into whole blood, indicating percent yield from 500 cells. n= number of separate healthy volunteers when >1 from same volunteer, the average was taken. As an internal control for the spiking results, ~500 EpCAM positive T47D breast cancer cells spiked into whole blood led to a percent recovery of nearly 100%.

As can be seen in **figure 5 and table 3,** in men with progressive mCRPC (n=8), we did not identify cellular events that expressed N-cadherin despite our ability to capture some positive control cells. Several explanations exist for these observations to date: 1) N-cadherin is expressed intracellularly rather than on the membrane and is not able to be captured by N-cadherin antibodies; 2) our N-cadherin antibody is unable to bind and capture membranous N-cadherin due to affinity or stability issues inherent in the processing; 3) N-cadherin expression is low in these CTCs collected at this time point. In order to sort out these issues, we are collecting N-cadherin-expressing CTCs over time in these men (baseline, cycle 3 at response, and at progression) in order to ascertain if N-cadherin expression increases during systemic chemotherapy (i.e. during EMT) and thus these cells may emerge during treatment. Indeed, we

have observed in one patient that the number of N-cadherin expressing CTCs increased from zero to eight during treatment, while the EpCAM positive CTCs declined during treatment. More data on this longitudinal assessment of N-cadherin expression will be collected during years 3-4. As stated above, in parallel, optimization of the N-cadherin antibody conjugation is ongoing as well with a variety of antibodies and positive control cell lines. Finally, we are examining the N-cadherin expression on CTCs capture using EpCAM-based methods through confocal microscopy to ascertain whether N-cadherin expression is localized to the membrane or is more cytoplasmic. This will be conducted in year 3.

For OB-cadherin, we were able to identify cellular events in men with progressive mCRPC (n=10). As shown in **figure 5** and the **table 3** below, in 4/10 cases, an equivalent or greater number of CTCs/events were captured using the OB-cadherin ferrofluid as compared to the standard EpCAM Cellsearch method. In two cases, these results were particularly dramatic, with 458 events identified with OB-cadherin vs. 45 events using the EpCAM Cellsearch method in one patient, and with 220 vs. 23 events respectively in a second patient. However, in other cases, the number of EpCAM + events was dramatically higher, with one patient having >1000 CTCs by the EpCAM Cellsearch method and only 3 by the OB-cadherin method, and another having 50 vs. 0 CTCs. Thus, we have observed widely ranging phenotypic heterogeneity among men with mCRPC. While all men have bone metastases, there appears to be a wide range in both EpCAM and OB-cadherin expressing cells in the bloodstream from these men. The number of OB-cadherin + cells in men with CRPC appears to be higher in many cases than observed in healthy volunteers (mean 72 vs. 4.4, median 3 vs. 0), indicating that these cellular events are tumor-related or tumor microenvironment related. We are currently conducting a range of molecular studies on these cells to further ascertain their malignant characteristics.

Table 3 Events captured from CRPC patients using each ferrofluid

Subject	N-cad capture		O-cad capture		EpCAM capture		CellSearch®
	# events	CD31+	# events	CD31+	# events	CD31+	standard-of-care
1	2	na	4	na	102	na	46
2	1	na	458	na	71	na	45
3	2	na	3	na	2	na	7
4	0	-	0	-	31	na	50
5	1	na	220	na	17	na	23
6	1	1	3	0	1351	na	>1000
7	na	na	1	0	0	-	1
8	2	0	0	-	53	1	22
9	0	-	29	29	9	0	12
10	na	na	2	1	111	1	69

The following studies in years 3-5 will be conducted to further characterize these cells. First, we will continue to optimize these antibodies and control cells in order to improve upon the recovery of these cells. Second, we will further characterize the reliability of these events as detected from patients using repeatability experiments. Third, we will evaluate how these N- and OB-cadherin + events change over time during response and progression. Fourth, we will further characterize these cells using a range of molecular probes against 1) the androgen receptor (AR), 2) TMPRSS2-ERG fusion genes (FISH break-apart assay), 3) PTEN loss by FISH, 4) centromeric probes to detect aneuploidy. Finally, confocal microscopy of human CTCs will be performed to determine cellular localization of N- and OB-cadherin. An example of this method on control PC-3 cells is shown in **figure 7**.

Task 4: To detect similarities in RNA expression profile and patterns of oncogenic expression in circulating tumor cells and matched metastatic prostate cancer tumor tissue (Months 1-54).

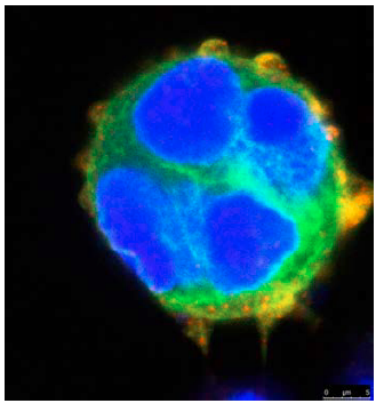


Figure 7. Confocal image of a PC3 cell stained for N-cadherin (red, Alexa 555) and Cytokeratin (green FITC), showing surface expression of N-cadherin and CK, using identical antibodies as used to capture or characterize cells in the modified CTC platform.

The major obstacle to performing RNA expression analysis from CTCs taken from patients with CRPC has been the isolation of a pure population of cells from whole blood that is devoid of leukocytes. Through a prolonged trial and error process intended to optimize this methodology, we have begun to have success in isolating these pure cellular populations. Our current operating procedures include drawing blood into EDTA vacutainers, followed by an initial red cell lysis followed by CD45 magnetic bead negative exclusion of contaminating leukocytes. This enriched CTC population is then further enriched and purified through FACS, gating against a tumor antigen (i.e. EpCAM) and negative excluding CD45 a second time. There is a high degree of CTC loss in this process; however, we are currently able to isolate a pure population of about 10-30% of the original spiked CTC population in simulated runs, which produces sufficient numbers of cells for RNA Sequencing.

At this time, we are working through the next step of taking these pure cells and performing RNA Sequencing after cell lysis and RNA preservation. We have a commitment from Illumina to collaborate on the testing of pure spiked and unspiked cell lines in healthy volunteer whole blood in order to determine if 1) quality RNA can be obtained from this, 2) to determine the RNA expression signature (noise) inherent in leukocytes as a negative control sample, and 3) to determine if a pure cellular tumor population can generate a reliable RNA expression profile from spiked blood over that of the background leukocytes. In these initial experiments, 10 and 50 cells will be sorted directly into lysis buffer, flash frozen, and then sent to Illumina for RNAseq using the single cell protocol. Each sample will be processed in duplicate. The objective of the first step is to determine the feasibility of this approach based on the quality of the RNA obtained. The objective of the second step is to determine whether distinct RNA expression patterns can be obtained from CTCs.

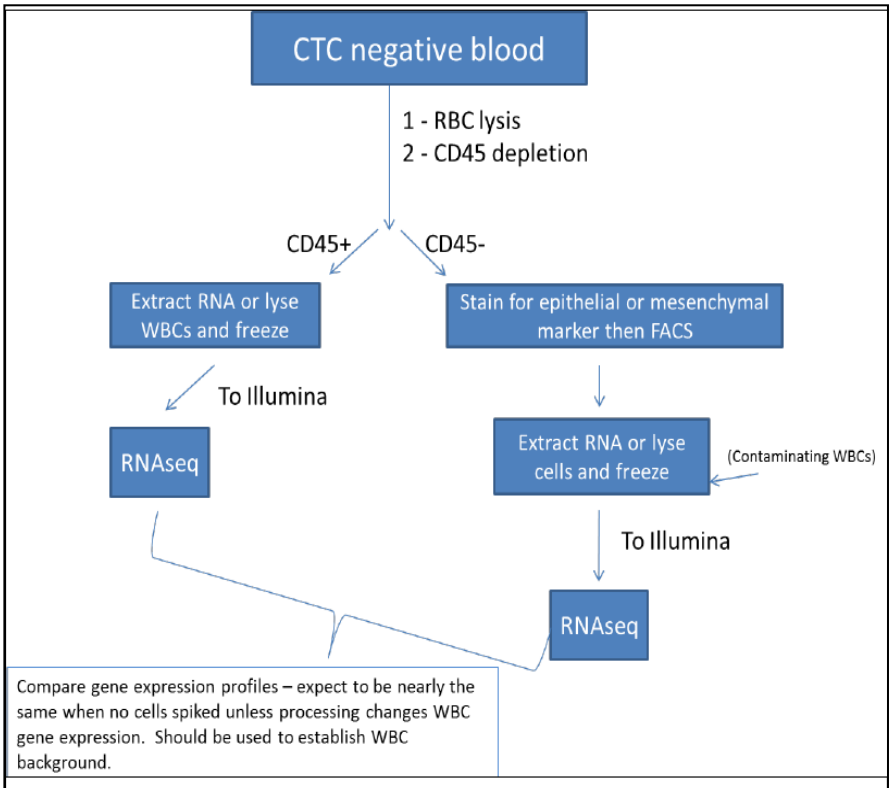


Figure 8. Whole blood without tumor cells spiked will initially be evaluated to determine the expected WBC signal as well as the effects of processing on the RNA quality.

Figures 8-9 detail the planned methodology during years 3-4 of this grant.

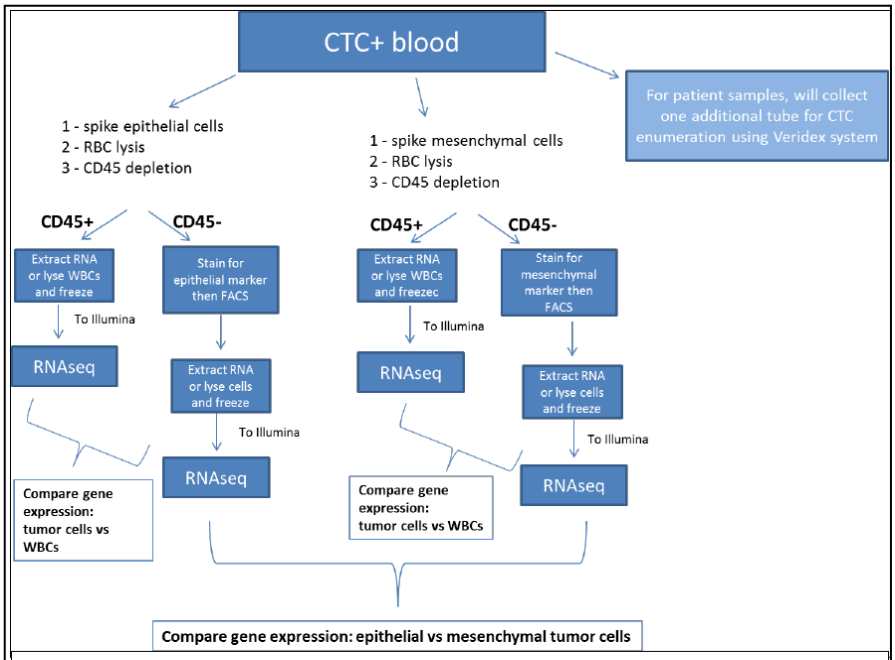


Figure 9. Both epithelial and mesenchymal tumor cells will be spiked into whole blood and recovered. The gene expression profiles between the recovered tumor cells will be compared to the WBCs from that sample. Gene expression of epithelial and mesenchymal cells will also be compared.

Following these preliminary spiking experiments of well annotated cell lines with known RNA profiles, we will be able to start performing RNA Sequencing from patients' CTCs isolated through a similar process but now from the clinic. We have Duke IRB approval already to conduct such work, but will not start collection of patient blood for RNA expression analysis until we have determined the success from these initial healthy volunteer blood spiking studies using control cell lines. Further funding for this work is being sought from multiple sources including the NIH through an R01 mechanism. The long term aim of this task is to identify molecular targets for personalized medicine approaches to therapy. In addition, we hope to gain insight into metastasis biology in prostate cancer. By examining the profiles of CTCs from patients in a moderate to large cohort of men, we can begin to assess the molecular drivers of cancer progression and mechanisms of dissemination. In addition, given that we have identified several populations of CTCs based on EMT biomarker expression, we should be able to isolate epithelial-like and mesenchymal-like CTCs and compare their RNA expression profile to understand the biology underpinning plasticity. Currently, the preliminary data for these projects and the initial optimization steps are being supported by Dr. Armstrong and the PRTA in terms of effort and technician support. No supplies/equipment for this task is provided by the PRTA.

Task 5: To estimate the clonality and heterogeneity of circulating tumor cells as compared with metastatic sites by DNA array-based comparative genomic hybridization (Months 1-54).

Key Research Accomplishments: In the past year, four microarray experiments have been run using DNA from T47D breast cancer cells and patient CTC and leukocyte cells on the Nimblegen Human CGH 4x72K Whole-Genome Tiling Array. T47D cells were selected as they have an established copy number profile and are thus suitable for feasibility/pilot studies and spiking experiments of cells lines into whole blood. Before hybridizing the samples on the microarray, the GenomePlex® Single Cell Whole Genome Amplification Kit (WGA4) was used to amplify the sample DNA to the yield required for microarray labeling. The WGA4 kit was used, because of excellent reproducibility and dependability of amplifying DNA from T47D and patient CTC and leukocytes.

We used T47D cells initially and performed FACS flow sorting followed by microarray. Before this microarray, we had optimized the methods for detecting T47D DNA diluted to be comparable to the amount of cells that we were expecting from the CTC patients (see year 1 report). The 4x72K microarray had four subarrays, with 72 thousand probes. The four subarrays contained the following samples: 1000 T47D cells without FACS flow sort, 1000 T47D cells with

FACS flow sort, 200 T27D cells with FACS flow sort, and 20 T47D cells with FACS flow sort. The results of the microarray are shown the in following **figure 10**.

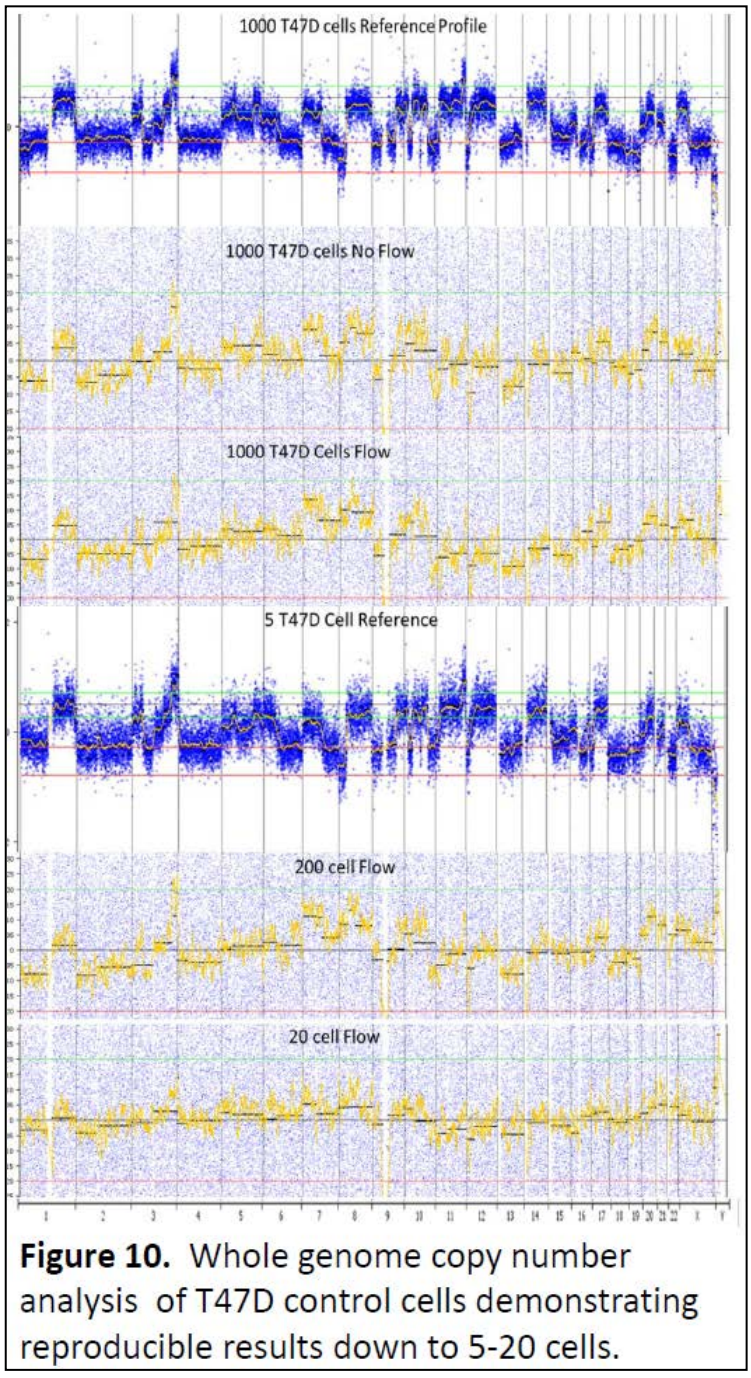


Figure 10. Whole genome copy number analysis of T47D control cells demonstrating reproducible results down to 5-20 cells.

The results showed a high degree of noise between the test and reference samples in all the arrays, but our Nexus Copy Number Software showed correlations in the breakpoints found in the reference T47D CNV profile and each of our microarray experiments. Also, we demonstrated for the first time that we could amplify and hybridize DNA that has been sorted in small numbers by a FACS sorter to a microarray.

Following this result, we proposed several solutions to fix the noise in the previous microarray experiment, as we believed the noise to be a result of over-amplification of the DNA sample with our WGA4 kit. For the next microarray we decided to label and hybridize a sample without using the WGA4 kit. T47D DNA was labeled and hybridized to the microarray. We did the same process for T47D cells that were flow sorted. We also sheared the T47D DNA to 250 bp fragments, because we thought it would allow for a more specific hybridization of the sample DNA to the microarray. In order to compare the control cell results with a patient sample, we performed this analysis on a man with metastatic progressive CRPC according to our Duke IRB approved protocol. This patient was known to have many CTCs, and thus was felt to offer a chance of observing copy number changes after CTC isolation from leukocytes. This patient's cells were RBC lysed and CD45 depleted and remaining EpCAM positive cells were flow sorted for microarray analysis. The second microarray experiment consisted of the following subarray samples: patient CTCs, 1000 unamplified T47D cells, 1000 amplified flow sorted T47D cells, and 1000 cells flow sorted and sheared. The results of the second microarray are shown in **figure 11**.

The results showed the unamplification and shearing brought the noise down considerably with the detection of control breakpoints remaining intact and similar to the previous array. These results confirmed that we could FACS cell sort, amplify and hybridize actual patient CTCs to a microarray. However, without internal control cells or external prostate cancer control cells, the interpretation of CTC arrayCGH data is challenging.

The third microarray experiment contained a microarray with 50 flow sorted T47D cells, 100 flow sorted T47D cells, and two patient CTC samples. We modified several methods in this experiment. With the WGA4, we ran amplification PCRs using 10 and 20 cycles in order to further reduce noise (false positive signals). We tried different amplification cycles to reduce the amount of overamplification. We labeled the samples twice as long as written in the Nimblegen protocol, from two hours to four hours. We hypothesized that the samples may have needed more time for efficient labeling. We also ran the hybridization at the max of 20 hours, to allow for complete hybridization to occur, which reduces the signal-to-noise ratio. We altered the microarray washing steps by heating the buffer solution to the 42 C to allow for the buffer salts to dissolve back into solution after crystalizing out of solution during the 4 C storage. After an aliquot was taken of the amount needed for the wash step, we allowed the working solution cooled to room temperature. The microarray was also washed and dried using twice the amount of time depicted in the protocol. In this experiment, we were able to reproduce a T47D CNV profile that almost perfectly matched our reference T47D CNV profile.

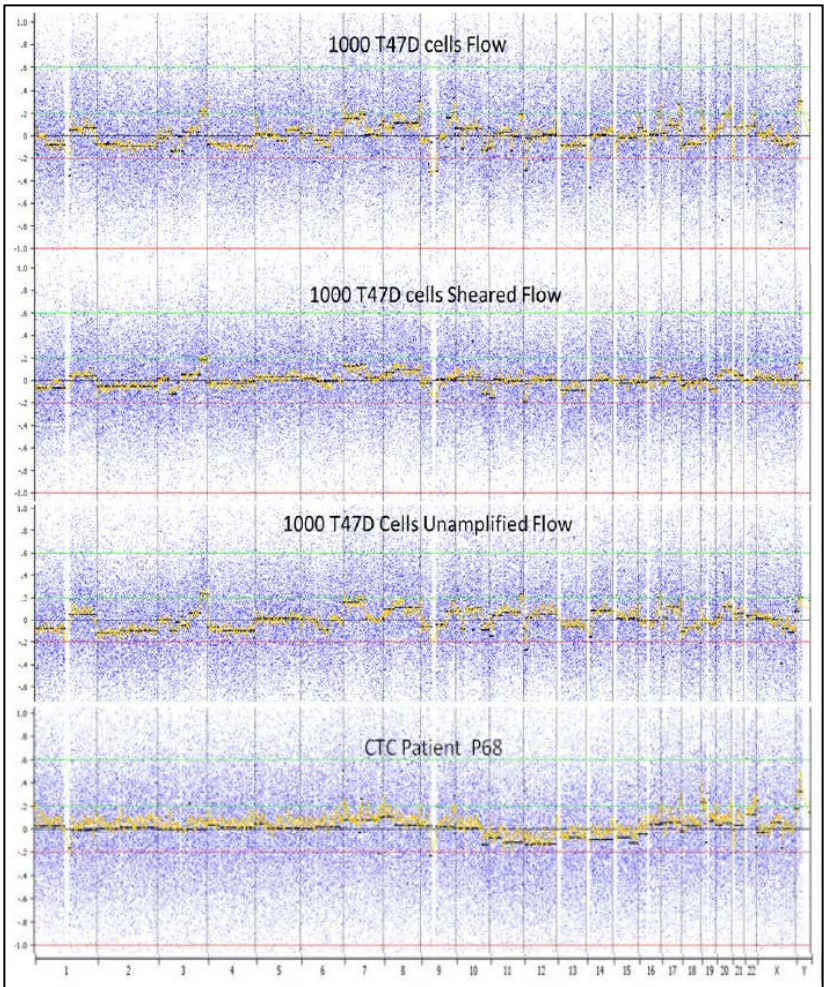


Figure 11. ArrayCGH results from our first CRPC patient (P68) demonstrating copy number analysis across the genome as compared with control cells.

Our CTC patient samples; however, did not closely resemble an annotated standard metastatic prostate cancer CNV profile[13]. An example of this is shown in **Figure 12**, where we compare the published and annotated copy number changes in metastatic prostate cancer with the results found from 2 of our patients with CRPC whose CTCs were analyzed for CGH. This may be related to technical factors such as biased amplification, contamination with normal leukocytes, or increased noise. In addition, without a comparison to a patient's own metastatic or primary tumor, it is challenging to validate any copy number changes observed in an individual's CTCs. As can be seen in chromosome 10, common loss of 10q23 (PTEN) is observed in the reference dataset for CRPC[13], but was not seen in either of our CRPC patient's CTCs. However, lack of correlation may be due to true differences in individual patient data as compared with the reference data, in which these patients may not have had PTEN loss in their CTCs. In general, however, we did not find common copy number gains or losses that have been consistently reported in CRPC (i.e. AR, PTEN, MYC, NCOA2, etc.), suggesting that our approach requires additional methodologic improvements in signal:noise detection.

For the next microarray, we proposed a number of different changes. First, we changed the reference DNA that was tested against the test sample from a CEPH family panel cell line, to the patient's own leukocytes. We thought this would provide more specific results, because we

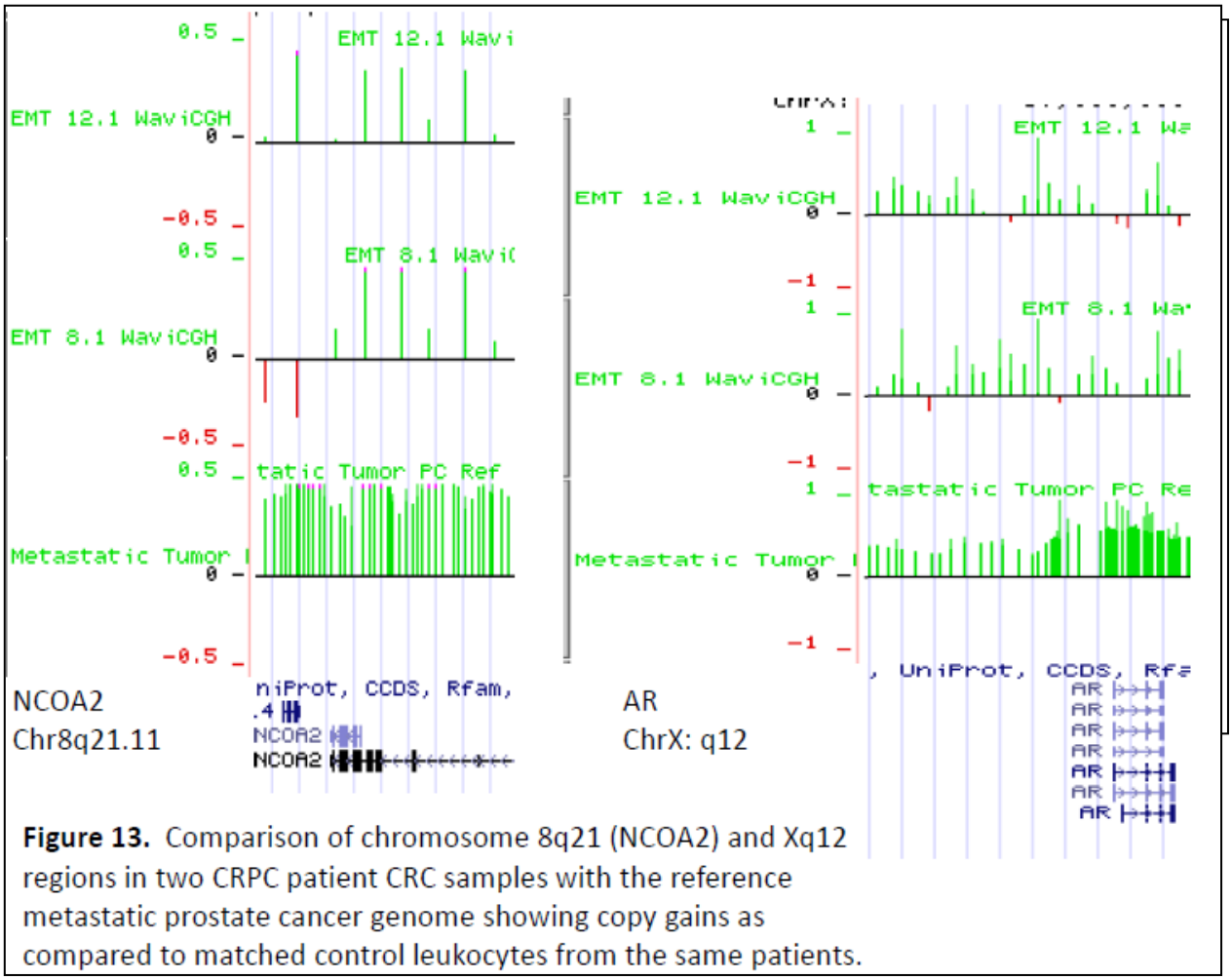


Figure 13. Comparison of chromosome 8q21 (NCOA2) and Xq12 regions in two CRPC patient CRC samples with the reference metastatic prostate cancer genome showing copy gains as compared to matched control leukocytes from the same patients.

were testing the tumor cell against its own DNA. We planned to make a duplicate test array of one of the patients. The duplicate was to depict reproducibility between the subarray experiments. We also ran a dye swap, where we switch the florescent dyes on one of the subarray samples. We run dye swaps to test the efficiency of the dyes by seeing if they give us the same results. We also planned that the next array would be completely patient CTCs, since we were able to reproduce the T47D CNV profile.

In the fourth microarray test, we used CTCs from two new CRPC patients. The first subarray contained patient CTCs matched against an amplified CEPH cell line. The second subarray contained patient CTCs against his own leukocytes. The third subarray was a duplicate of the second subarray. The fourth subarray was also the same as the second subarray; however, the CTCs used the Cy5 dye and the leukocytes used the Cy3 dye. The results are presented in the **figure 13** below. In this figure, we have highlighted two common copy variant regions in the prostate cancer genome at 8q24 (myc) and Xq12 (AR). Our data confirmed the reliability of our CGH assessments using the dye swap (data not shown) and using duplicate samples. In addition, we are starting to now observe copy number changes in the CTCs from patients that have been validated externally in the prostate cancer genome dataset.

These results suggest that the microarray experiment is being performed in a reproducible manner. The observation of copy gains, even a single copy gain, of AR and NCOA2, suggests that we may be able to detect common chromosomal derangements in the CTCs from patients with metastatic cancer. NCOA2 is a nuclear receptor (AR) co-activator and copy gains have been found in 24-37% of metastatic CRPC patients and NCOA2 is involved in AR signaling, suggesting biologic relevance to CRPC progression[13]. These results suggest that important pathophysiologic changes in the DNA of prostate cancer cells may be detectable in the CTCs of

men with metastatic CRPC in real time, rather than on tissue derived from autopsy or biopsy studies. More patient samples and a broader comparison of key prostate cancer copy changes across the genome are needed, and will be completed in the next years of the grant.

One reason for the low signal intensity from CRPC CTCs could be the difference in the amount of DNA copies between the patient CTC and leukocyte DNA. For example, if there are many fewer CTC DNA copies to leukocytes during the WGA4 amplification, the amount if leukocytes DNA copies will skew the CNV profile result. For the next array during year 3, we plan to ensure that we are using an equal amount of CTC cells and leukocyte cells before the WGA4 amplification. Additional patient samples will then be analyzed. DOD PRTA funds continue support the effort of Dr. Armstrong and one technician for this work but do not support the actual supplies. We plan to apply for additional funding for this work once the methods have been optimized and sufficient compelling preliminary data has been generated for an R01 or R21 NIH grant or other DOD-funded grant.

Task 6: To develop skills necessary to succeed as a leader in clinical and translational research and obtain independent peer-reviewed research funding to support a long term career in research (Months 1-60)

During year 2, Dr. Armstrong enrolled in the Duke Clinical Research Training Program (CRTP) and completed 2 courses successfully in proteomics and Clinical Research Management. Skills obtained from these courses will further his research aims in the investigation of protein biology and translational clinical trial conduct. He plans to complete one CRTP course per semester for the next 2-3 years of the PRTA grant based on course availability and relevance to his ongoing research.

Dr Armstrong is a full Duke IRB board member and a member of the Duke Cancer Institute's Cancer Protocol Committee, which provides scientific review pre-IRB review for cancer protocols. This practice-based training in clinical research is ongoing and provides much needed experiential education in the conduct of clinical trials and research design and safety oversight.

Dr. Armstrong was promoted in January of 2012 to Associate Professor of Medicine, recognition for the success in publications and academic achievement facilitated by this current DOD PRTA. He was also appointed as the co-director for clinical and translational research in GU oncology within the newly formed Duke Cancer Institute, overseeing the clinical trial and translational aspects of research in prostate cancer and other GU malignancies at Duke.

Dr. Armstrong participates regularly in a number of educational and research based meetings at Duke, including the prostate cancer journal club (monthly), clinical research staff meetings (oversight of trial conduct, data and safety monitoring, weekly), the Garcia-Blanco laboratory meetings (weekly to monthly), the prostate cancer pre-SPORE meetings (monthly), new protocol meetings (to review internal and external proposals, monthly), Translational Science meetings through the Department of Pharmacology and Cell Biology (monthly), Oncology Grand Rounds (weekly), urology tumor board (monthly). Dr. Armstrong participated in 2010 in a Duke Clinical Research Leadership Retreat (3 days), sponsored by the Dean's Office, which provided valuable feedback and education in a range of leadership topics from conflict resolution to giving feedback to leading a diverse team of staff and peers. Dr. Armstrong serves on the oncology fellowship committee, and is now a mentor to a medical oncology fellow and emerging faculty member (Rhonda Bitting), who is also working on these CTC biology projects. He also served as a mentor to a Duke undergraduate (Avian Ettyreddy) in the Garcia-Blanco lab who is working on a project related to the development of antibodies against FGFR2 splice variants that will allow for the detection of these splice variants in CTCs and human cancer samples. He serves as a clinical mentor to two post-doctoral fellows in the Garcia-Blanco laboratory, Drs. Jason

Somarelli and Daneen Schaeffer, each of whom works on cancer metastasis and plasticity biology as it relates to FGFR2 signaling and alternative splicing.

In addition, Dr Armstrong participates in the following national-level programs that provide training in leadership and clinical practice: ASCO (Chair of a Clinical Science Symposium in 2012), NCCN (Prostate Cancer guidelines member), GU Symposium (ASCO-ASTRO-SUO), and the Prostate Cancer Foundation annual scientific retreat. He is co-chair of the 2012 PCF Retreat Session on EMT in Prostate Cancer. He attended the DoD IMPACT meeting in 2011 as well and presented an educational talk on "Prostate Cancer 101" in addition to presenting several posters and leading a walking tour of the posters. In year 3, Dr. Armstrong will continue with these educational and scientific endeavors as part of his clinical research training and academic mission.

KEY RESEARCH ACCOMPLISHMENTS—YEAR 2 (See Year 1 for additional accomplishments)

- **Accepted manuscript** in Molecular Cancer Research (Armstrong et al, attached, published online first June 10, 2011) related to task 2. This data presented the strongest evidence to date for the existence of EMT in human cancer, including men with metastatic prostate cancer and women with metastatic breast cancer. This paper was highlighted in a number of press releases and journals.
- Development of **methods** to isolate individual circulating tumor cells for genetic and genomic analyses (tasks 4-5) and to optimize single cell arrayCGH profiling of rare cells
- Attainment of **IRB approval** at the Durham VAMC (task 1) and the Duke University IRB (tasks 2-3) along with continuing reviews
- **Validation** of tissue microarray linked to SEARCH database using a control biomarker (Ki-67) that will allow for interpretation of the relationship between EMT biomarkers in surgical prostatectomy specimens to be ascertained and correlated with clinical outcome
- Ongoing training in clinical research and clinical oncology as stated above in task 6, with successful application to the Duke Clinical Research Training Program for ongoing clinical research training.
- Development of a **patent** focused on the novel detection of circulating tumor cells using an EMT antigen-based ferromagnetic capture method (submitted 9/24/10, international application number PCT/US10/50223).
- Development of a **research collaboration agreement** with Veridex/Johnson and Johnson towards the development of a novel CTC capture method based on these findings using N-cadherin or O-cadherin based ferrofluids to capture CTCs from the blood. This work is ongoing.
- Presentation of data at national meeting: AACR meeting on EMT (Washington, DC February 2010 invited speaker and poster presenter) entitled "Circulating Tumor Cells from Patients with Metastatic Breast and Prostate Cancer Express Vimentin and N-Cadherin".
- Presentation of data at national meeting: ASCO 2012. "Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method". See attached poster and abstract[14].

REPORTABLE OUTCOMES

1) **Manuscript: Armstrong** et al, Molecular Cancer Research 2011 (online 6/10/11, see appendix)
2) **US Patent:** submitted 9/24/10 for the detection of circulating tumor cells based on EMT antigen-based capture
3) **Research Collaboration Agreement:** with Veridex/J&J to develop a next-generation CTC detection assay based on our preliminary data and published data from this PRTA. This has included the provision and development of a research Cellsearch® machine and Cell tracks® Analyzer and two novel ferrofluids for clinical testing.
4) **ASCO GU Symposium poster/abstract 2010 (abstract 172)**
5) **Oral podium presentation, AACR Conference on EMT 2010 (Washington, DC)**
6) **Presentation of data at national meeting: ASCO 2012. "Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method". See attached poster and abstract.**
7) **Development of Tissue Microarray and validation with SEARCH Database (Durham VA Medical Center)**
8) **Development of methods for single cell isolation and arrayCGH DNA profiling from whole blood**
9) **Grant proposals that arose from this award:** Sidney Kimmel Scholar Award (not funded), Provocative Question NIH R01 (multi-PI, not awarded 11/2011), U01 (PI: Dewhirst) award for Tumor Microenvironment research (pending)
10) **Acceptance to Duke CRTP program, Summer 2011, ongoing**
11) **Publication of review article: "Biomarkers in the management and treatment of men with metastatic castration-resistant prostate cancer." European Urology 2011[15].**
12) Abstract 10533, ASCO 2012. "Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method". See attached poster and abstract in appendix[14].

CONCLUSIONS

In this second annual report for the DOD PRTA, we have provided strong evidence for the existence of epithelial-mesenchymal transition in human cancer, particularly prostate cancer. These findings are based on the discovery of EMT and stemness biomarker expression in CTCs from men with CRPC and for the existence of a novel CTC phenotype based on EMT-biomarker capture. The findings of EMT and stemness markers in the majority of circulating tumor cells from men with metastatic prostate cancer and women with metastatic breast cancer has several important implications: 1) EMT and stemness pathways likely play an important role in cancer progression, metastasis, and lethality and suggests routes for new therapeutic approaches; and 2) current methods to detect CTCs that rely solely on epithelial characteristics (i.e. EpCAM) may miss a large number of non-epithelial tumor cells that have undergone EMT during circulatory spread and transit. We intend to apply our findings directly toward therapeutic target identification and technology development. In order to accomplish this, we have made significant progress in developing methods for whole genome DNA analysis of rare cells and have begun to apply this copy number analysis to circulating tumor cells from men with metastatic CRPC. We will continue to optimize our methods for single cell (CTC) capture and isolation from whole blood for DNA and RNA profiling, which will allow us to profile these cells in real time from patients and to compare the RNA and DNA characteristics of CTCs captured using both epithelial and mesenchymal antigen retrieval methods. Given that many cells may be missed by focusing only on EpCAM capture alone, these techniques may be able to identify the phenotype and molecular targets in a novel CTC population. By identifying novel cell phenotypes and pathways that contribute to lethal cancer progression, our hope is to use these CTC biomarkers early to detect and thus intervene with novel therapeutic approaches to prevent or significantly delay metastasis in prostate cancer.

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Appendices

A. ASCO 2012 Abstract and poster

B. Manuscript (Armstrong et al, Molecular Cancer Research 2011)

C. Current CV

Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method

Rhonda L. Bitting, Rengasamy Boominathan, Chandra Rao, Elizabeth Embree, Daniel J. George, Mark Connelly, Gabor Kemeny, Mariano A. Garcia-Blanco, and Andrew J. Armstrong

Background

Circulating tumor cells (CTCs) have potential prognostic, predictive and surrogate implications in oncology. In patients with metastatic castration-resistant prostate cancer (CRPC) and breast cancer (BC), we have shown that CTCs isolated using epithelial cell adhesion molecule (EpCAM) ferromagnetic capture express mesenchymal markers, including N- and O-cadherin, suggesting phenotypic plasticity and the presence of epithelial-mesenchymal transitions (EMT). Therefore, we postulate that during metastasis, tumor cells exist as a spectrum of epithelial to mesenchymal phenotypes and may not be captured with existing EpCAM-based CTC technology. The goal of this study is to identify CTCs in CRPC and BC patients using a novel mesenchymal-based capture method.

Methods

In patients with advanced CRPC and BC, two EDTA and one CellSave® tube of blood are collected. Using the CellSearch® system (Veridex, USA), CTCs are captured with either anti-N-cadherin (N-cad) or anti-O-cadherin (O-cad) ferrofluid and detected cells (events) are defined as beta-catenin and DAPI positive, CD45 negative intact cells. We evaluated the performance of these EMT-based ferrofluids in healthy volunteers, control cells, and in a pilot study of patients with CRPC, and compared enumeration of cells using novel vs. standard EpCAM-based capture methods.

Results

In healthy volunteers, rare events were detected using the novel capture methods. In CRPC patients, O-cad capture detected more events in 3 of 5 subjects than EpCAM-based capture, and the majority of captured cells were cytokeratin negative. See table:

	O-cad capture, beta-catenin+	N-cad capture, beta-catenin+	EpCAM capture, cytokeratin+
Healthy volunteers	0-51 events (mean 5.95) n=21	0-4 events (mean 0.28) n=25	NA
CRPC patients	0-465 events (mean 138.4) n=5	0-2 events (mean 1.2) n=5	1-123 CTCs (mean 50.8) n=5

Conclusions

These preliminary results suggest the existence of a novel CTC phenotype based on EMT properties in CRPC, particularly overexpression of O-cadherin. Further characterization of these cells from patients with advanced PC, BC, and other solid tumors will provide insight into EMT and metastasis biology.

Isolation of Circulating Tumor Cells Using a Novel EMT-Based Capture Method

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Background

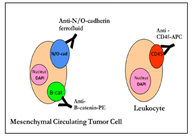
Circulating tumor cells (CTCs) have potential prognostic, predictive and surrogate implications in oncology. In patients with metastatic castration-resistant prostate cancer (CRPC) and breast cancer (BC), we have shown that CTCs isolated using epithelial cell adhesion molecule (EpCAM) ferromagnetic capture express mesenchymal and stem cell markers, including N- and O-cadherin, suggesting phenotypic plasticity and the presence of epithelial-mesenchymal transitions (EMT).[1] Therefore, we postulate that during metastasis, tumor cells exist as a spectrum of epithelial to mesenchymal phenotypes and may not be sufficiently captured with existing EpCAM-based CTC technologies.

Objective

To identify CTCs in CRPC patients using a novel mesenchymal-based capture method and to evaluate the performance of these novel cell capture methods in healthy volunteers.

Methods

- Patients with progressive, metastatic CRPC are enrolled prior to initiating a new systemic therapy.
- One CellSave® and two EDTA 10 mL tubes of blood are collected at baseline, at treatment cycle 3, and at progression.
- Using the CellSearch® system (Veridex, USA), CTCs are captured with anti-N-cadherin (N-cad) (Santa Cruz D4) or anti-O-cadherin (O-cad) (R&D Systems 283416) ferrofluid, then permeabilized and stained for further characterization.
- Mesenchymal CTCs (events) are defined as β-catenin+, CD45-, nucleated intact cells, based on the importance of β-catenin in EMT and on our preliminary data that β-catenin is absent in leukocytes.
- Performance evaluated in healthy volunteers, control cells (PC3, BT549, osteoblasts), and in a pilot study of patients with metastatic CRPC.
- Mesenchymal CTC enumeration compared with the standard EpCAM-based capture method.[2]



Results

- 60-90% of EpCAM-captured CTCs from CPRC patients express β-catenin (Figure 1).
- Positive control tumor cells also express β-catenin.
- Here we use β-catenin expression in place of cytokeratin expression to identify mesenchymal CTCs.

Figure 1. Examples of β-catenin expression in EpCAM-captured CTCs

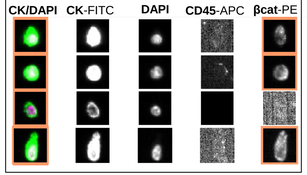
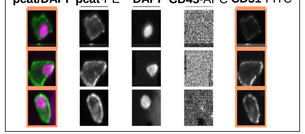


Figure 2. Examples of O-cadherin-captured events from healthy volunteers



- Rare O-cadherin-captured, β-catenin+ events are detected in healthy volunteers (HVs).
- Detected events stained with the endothelial marker CD31 for further characterization.[3]
- In HVs,100% of events tested to date are CD31+ (Figure 2).

Table 1. Baseline characteristics of CRPC patients

	Results (n=10)
Median age, y (range)	68 (57-74)
Race	
Caucasian, n (%)	7 (70)
Black, n (%)	3 (30)
Karnofsky performance status, median (range)	90 (80-100)
Gleason score, median (range)	8 (7-10)
Pain score >4, n (%)	4 (40)
Initial local therapy	
Prostatectomy, n (%)	3 (30)
External beam radiation, n (%)	3 (30)
None, n (%)	4 (40)
Laboratory values	
PSA ng/mL, median (range)	408 (7-4377)
LDH U/L, median (range)	220 (206-291)
Hemoglobin g/dL, median (range)	9.8 (8.8-12.1)
Alkaline phosphate U/L, median (range)	197 (57-463)
CTC count, median (range)	34 (1-1000)
Sites of metastasis	
Bone, n (%)	10 (100)
Liver, n (%)	2 (20)
Lung, n (%)	4 (40)
Lymph nodes only	0
Prior therapies	
Number of hormonal therapies, median (range)	4 (1-5)
Abiraterone, MDV3100, or TAK700, n (%)	7 (70)
Sipuleucel-T, n (%)	3 (30)
Docetaxel, n (%)	8 (80)
Cabazitaxel, n (%)	2 (20)
>1 chemotherapy, n (%)	2 (20)
Bone targeted therapy, n (%)	9 (90)
Palliative radiation, n (%)	3 (30)
Type of progression prior to study enrollment	
Imaging	8 (80)
Clinical (symptoms, PSA increase)	2 (20)

Results

Figure 3. Events captured from healthy volunteers and CRPC patients using each ferrofluid

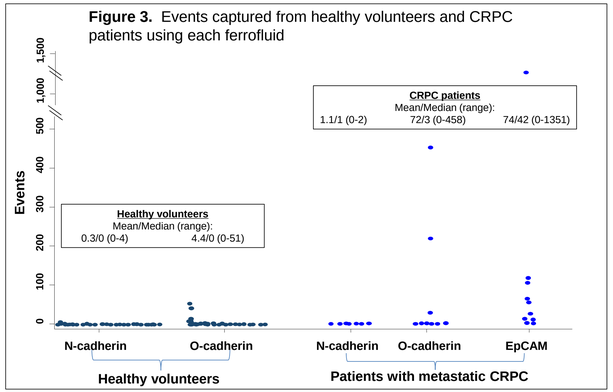
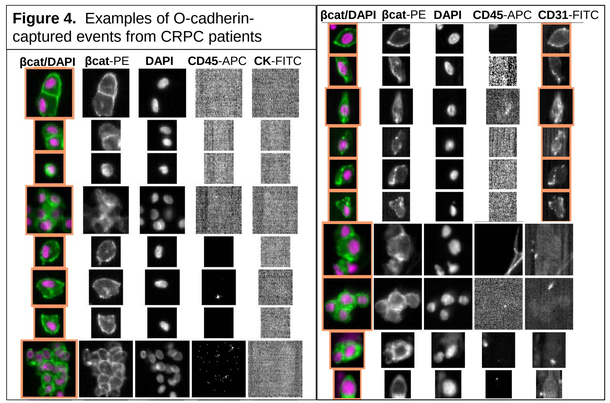


Figure 4. Examples of O-cadherin-captured events from CRPC patients



Results

Table 2. Events captured from CRPC patients using each ferrofluid

Subject	N-cad capture		O-cad capture		EpCAM capture		CellSearch®
	# events	CD31+	# events	CD31+	# events	CD31+	standard-of-care
1	2	na	4	na	102	na	46
2	1	na	458	na	71	na	45
3	2	na	3	na	2	na	7
4	0	-	0	-	31	na	50
5	1	na	220	na	17	na	23
6	1	1	3	0	1351	na	>1000
7	na	na	1	0	0	-	1
8	2	0	0	-	53	1	22
9	0	-	29	29	9	0	12
10	na	na	2	1	111	1	69

Conclusions

- These preliminary results suggest that O-cadherin and β-catenin positive cellular events are detectable in men with mCRPC and are less common in healthy volunteers.
- O-cadherin events frequently express the endothelial marker CD31.
- Further molecular characterization needed to determine if these CD31+ events are endothelial cells, circulating tumor microenvironment, or tumor cells with features of vasculogenic mimicry.[4]
- Fluorescence *in situ* hybridization for cell ploidy, TMPRSS2-ERG status, PTEN loss, and AR amplification[5] is ongoing to determine if cellular events are tumor-derived.
- CTCs and O/N-cadherin captured events are collected at baseline, while on treatment, and at progression to determine if phenotypic changes emerge over time.
- Further optimization of the N-cadherin capture method is ongoing.

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Molecular Cancer Research

Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers

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Angiogenesis, Metastasis, and the Cellular Microenvironment

Molecular Cancer Research

Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers

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Abstract

During cancer progression, malignant cells undergo epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET) as part of a broad invasion and metastasis program. We previously observed MET events among lung metastases in a preclinical model of prostate adenocarcinoma that suggested a relationship between epithelial plasticity and metastatic spread. We thus sought to translate these findings into clinical evidence by examining the existence of EMT in circulating tumor cells (CTC) from patients with progressive metastatic solid tumors, with a focus on men with castration-resistant prostate cancer (CRPC) and women with metastatic breast cancer. We showed that the majority (>80%) of these CTCs in patients with metastatic CRPC coexpress epithelial proteins such as epithelial cell adhesion molecule (EpCAM), cytokeratins (CK), and E-cadherin, with mesenchymal proteins including vimentin, N-cadherin and O-cadherin, and the stem cell marker CD133. Equally, we found that more than 75% of CTCs from women with metastatic breast cancer coexpress CK, vimentin, and N-cadherin. The existence and high frequency of these CTCs coexpressing epithelial, mesenchymal, and stem cell markers in patients with progressive metastases has important implications for the application and interpretation of approved methods to detect CTCs. *Mol Cancer Res; 9(8); 1–11. ©2011 AACR.*

Introduction

Most metazoan cells can be classified as either epithelial or mesenchymal based on morphology, behavior, and molecular signatures. In adult animals, epithelial and mesenchymal cells usually remain in one phenotypic state; that is, epithelial cells do not change their properties and become mesenchymal. During development, however, epithelial cells of the early embryo give rise to all 3 embryonal layers (endoderm, mesoderm, and ectoderm), which include mesenchymal cells (1). Therefore, these early embryonal cells have the ability to transition between epithelial and mesenchymal states, a property we define as epithelial plasticity (for a slightly different definition see ref. 2). Indeed, observations in embryos showed epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET; ref. 3), which may be viewed, perhaps naively, as forward and reverse directions of the same reaction mechanism (for review, see refs. 2, 4).

Although it is convenient to present EMT/MET as a reversible reaction between binary states, there are suggestions that intermediate states exist and that these may play important roles. The importance of EMT and MET in cancer progression is now widely, albeit not unanimously, accepted (see recent reviews refs. 4–8). EMT is observed in human cancer cells *in vitro* and in xenografts, and in the leading edge of invasive carcinomas *in vivo* (5, 6). In human prostate carcinoma, loss of E-cadherin expression and overexpression of N-cadherin, which indicates the presence of an EMT, independently correlates with high Gleason score and systemic and metastatic recurrence after surgery, linking EMT to more aggressive clinical behavior (9–12). In addition, recent studies have shown the importance of

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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N-cadherin expression during castration-resistant metastatic progression in preclinical models of prostate cancer and in human metastases. These translational studies have suggested a link between loss of epithelial markers, gain of mesenchymal markers, and the induction of signaling pathways that promote survival and androgen-receptor independent growth (13). In breast cancer, a similar link has been established between EMT markers in primary and disseminated bone marrow tumor cells and aggressive clinical behavior (14–18). Likewise, evidence for MET was obtained from microscopic analysis of colorectal carcinoma metastases, which adopted epithelial characteristics of the noninvasive regions of the primary tumor (19). In prostate cancer, attachment of metastatic cells to bone cells correlates with expression of E-cadherin (20). These and many other studies describe the existence of these transitions during carcinogenesis and raise questions about their functional importance. There is strong evidence that EMT is important for metastatic behavior and chemoresistance (18, 21); however, the importance of MET has been more difficult to ascertain. Previously, we found that the preponderance of MET events among lung metastases in rats bearing AT3 rat prostate adenocarcinoma tumors suggested an important functional relationship between the capacity to revert to a more epithelial state and metastatic growth in the lung parenchyma (22, 23).

A strict view of epithelial plasticity in cancer posits that a mesenchymal-like state reached post-EMT is the driver of malignant fitness. Indeed, there is strong evidence that the mesenchymal properties of invasiveness and motility are required for metastases (see above) and that EMT leads to expression of cancer stem cell markers, including CD44 (24). Nonetheless, observations above suggest that mesenchymal properties per se are not sufficient for optimal malignant behavior (19, 22, 23, 25). A broader interpretation suggests that the ability to easily transition between epithelial-like and mesenchymal-like states, which we define as phenotypic plasticity, may be linked to stem cell–like properties and is a more important determinant of aggressive metastatic behavior than the properties of the end states. In a preclinical model examining the importance of stem cells and cancer growth, subcutaneous injection of CD133-positive cells, which are predicted to be stem cell like, into immunodeficient mice causes tumor growth, whereas injection of CD133-negative cells do not (26). A recent clinical study that measured mRNAs coding for stem-like markers in the bloodstream of patients with resected colorectal cancer found that the expression of CD133, CEA, and cytokeratin (CK) RNAs was associated with recurrent disease and an overall poor prognosis (27).

Therefore, we posit that the most plastic cells will be those that inhabit transitional or intermediate states with properties of both epithelium and mesenchyme, and that these transitional cells will be particularly malignant and stem-like. To test this proposal in human disease, we sought evidence for markers of both mesenchymal and stemness phenotypes in circulating tumor cells (CTC) from patients with metastatic castration-resistant prostate cancer (CRPC) and metastatic breast cancer.

Materials and Methods

Analysis of human CTCs

Patients eligible for the CTC biomarker protocols included (i) men with mCRPC, with metastatic progression by prostate-specific antigen (PSA; 2 consecutive rises over nadir separated by more than 1 week) or radiologic criteria (Response Evaluation Criteria in Solid Tumors or new bone scan lesions), a PSA 5 or more, age 18 years or older; or (ii) women with metastatic breast cancer with disease progression or with initiation of a new systemic therapy, who were older than 18 years of age, and who were at least 7 days from treatment with an anthracycline-containing regimen. All subjects provided informed consent as part of an Institutional Review Board-approved prospective clinical protocol. Blood (15 mL) was collected from patients and processed within 48 hours at the Duke University, Department of Molecular Pathology and Clinical Pathology Laboratory using the Cell Search System (Veridex, Raritan). Veridex profile kits, which isolate epithelial cell adhesion molecule (EpCAM)-positive cells by using a ferromagnetic immunoabsorbtion assay without additional staining, were used to collect CTCs. An additional tube was collected and processed in parallel for CTC enumeration by using the Veridex CellSearch method using the standard test kit. Following profile kit processing, the isolated cells were either processed immediately or stored overnight in 4% paraformaldehyde (PFA) and processed the next day. An initial wash using a bench top magnet to enrich the EpCAM-bound cells was conducted to further isolate CTCs, with resuspension of the cell pellet after magnet release into 100 μL PBS. Immunostaining was done on teflon-coated slides. Briefly, cells were pipetted into the wells of the slides and left to settle for ∼30 minutes followed by standard immunostaining procedures with careful aspiration to minimize cell loss at room temperature. Following 4% PFA fixation, permeabilization with PBT (PBS with 0.2% v/v Triton), and blocking with 10% goat serum for 30 minutes, triple immunostaining was done using CD45 antibody labeled with Alexa 647, CK labeled with Alexa 555 and either Vimentin (BD Biosciences), N-cadherin (BD Biosciences), O-cadherin (Invitrogen), or CD133 (Novus Biologics) labeled with Alexa 488. Supplementary Table S1 provides specifics on the control cells, dilutions, and products used. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) was then done. A CTC was defined as an intact cell, containing a nucleus and expressing CK but lacking CD45 expression. Control cells were evaluated in parallel with each patient sample for immunofluorescent staining intensity and scoring (see Supplementary Table S1 for controls used and Supplementary Fig. S1 for images of these real-time controls). Human peripheral blood mononuclear cells (PBMC), obtained by Ficoll purification of buffy coats from normal American

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Red Cross donors, were kindly provided by Micah Luftig (Duke) and used as positive control cells for CD45 expression and negative controls for CK and cadherin proteins. In addition, the coexpression of E-cadherin (BD Biosciences) with N-cadherin on EpCAM-captured, CD45-negative DAPI-positive cells, irrespective of their CK expression, was examined. In this situation, all CD45-negative cells were manually enumerated and the proportion of CD45-negative nucleated cells that expressed E- or N-cadherin were scored. To ensure that the antibodies against E- and N-cadherin (Supplementary Table S1) did not cross-react with their respective antigens, a series of control cells with known E- and N-cadherin expression levels were examined by Western blot analysis against E- and N-cadherin and actin as a loading control.

The slides were mounted with gel/mount media (Biomeda). The slides were analyzed with an Olympus IX 71 epifluorescence microscope, and images were acquired using an Olympus DP70 digital camera. Image processing was done with DP Controller software (Olympus). All fields on each slide were analyzed sequentially, with each CK-positive–nucleated cell that was CD45 negative being counted as a CTC. Standardized exposure times optimized for each antibody were used consistently throughout the analysis for case and control cells.

Immunohistochemical analysis of metastases

Under the same informed consent protocol, men undergoing CTC collection additionally consented to have a radiologic-guided metastatic biopsy for analysis of biomarker expression by immunohistochemistry (IHC). Samples were obtained through core needle biopsies during light sedation, and immediately formalin fixed and paraffin embedded. For analysis, slides were deparaffinized, rehydrated, and endogenous peroxidase was inactivated for 30 minutes in 0.3% H_2O_2 (hydrogen peroxide) in methanol. Specific antigen retrieval steps were done for individual antigens. Three markers were evaluated by IHC: vimentin (M7020; Dako, 1:150; antigen retrieval with pepsin treatment at 37°C for 15 minutes), CK cocktail (18-0132; Invitrogen, 1:50 and 349205; BD Biosciences 1:50, antigen retrieval with pepsin treatment at 37°C for 15 minutes) and CD45 (M0701; Dako, 1:200; antigen retrieval with sodium citrate 10 mmol/L, pH 6.0 at 100°C for 30 minutes). Primary antibody was incubated for 60 minutes at room temperature. Dako Envision horseradish peroxidase secondary antibody was used for 30 minutes at room temperature and the signal was detected with 3,3′-diaminobenzidine reagent (Vector kit SK 4100). Slides were counter stained with hematoxylin and eosin and assessed by a trained pathologist for expression using appropriate positive (localized prostate tissue microarray sections) and negative controls (mock antibody) for each marker.

Statistical analyses

We used simple descriptive statistics to estimate the prevalence of mesenchymal and CD133 antigen coexpression on EpCAM-captured CTCs, summarizing these findings on both an intraindividual level and within group (CRPC and breast cancer) level. To compare CTC count (standard CellSearch method) against the proportion of CTCs that coexpress vimentin, N- and O-cadherin, or CD133, linear regression analysis was done. Goodness of fit was tested by ANOVA.

Results

To examine the coexpression of mesenchymal and/or stemness antigens on CTCs from patients with metastatic disease, we took advantage of the existing Food and Drug Administration (FDA)-approved capture method to initially capture and isolate cells from whole blood. CTCs have both independent prognostic and predictive significance in multiple epithelial malignancies, including mCRPC and metastatic breast cancer (28, 29), and can be collected, isolated, and analyzed for a variety of biomarkers relevant to cancer biology (30–32). The approved technology for CTC capture relies on the expression of EpCAM on the surface of epithelial cells, and thus currently measured CTCs must be epithelial like. Yet, these cells have escaped from the primary tumor, possibly as a result of an EMT/invasiveness program, and may have mesenchymal properties.

To test for the existence of mesenchymal-like CTCs, blood was collected from 41 men with mCRPC and 16 women with metastatic breast cancer (see baseline characteristics for the patients in Table 1 and Supplementary Table S2) and CTCs were processed by using the CellSearch EpCAM-based immunocapture method and profiled for expression of CD45 (PTPRC) (a leukocyte marker), CK, and E-cadherin (CDH1; epithelial markers), vimentin (VIM), N-cadherin (CDH2), and O-cadherin (CDH11; mesenchymal markers), and CD133 (a stem cell marker) by immunofluorescence (IF; refs. 10, 33; Supplementary Table S1). Leukocytes were defined as nucleated (DAPI positive), CD45-positive and CK-negative cells (Fig. 1A), whereas CTCs were defined as nucleated (DAPI positive), CD45-negative and CK-positive cells (Fig. 1B–G). Among CTCs, we identified subsets of cells that additionally expressed vimentin (Fig. 1C) or N-cadherin (Fig. 1D and E) or O-cadherin (Fig. 1F and G). Real-time positive and negative control cells were used at the time of individual patient CTC analysis to determine positive or negative IF expression using identical exposure, antibody concentration, and camera settings without alteration. Real-time control cell images for each corresponding antigen and subject depicted in Figures 1, 3, and 4 are provided in Supplementary Figure S1.

Among men with mCRPC, we found that CTCs coexpressed vimentin and CK in 10/10 (100%) patients, and by this criterion 108/126 (86%) of enumerated CTCs coexpressed these markers (Fig. 1B–C, Table 2 and Supplementary Table S3, and also see Supplementary Fig. S1 for control cells and Supplementary Fig. S2 for additional examples). Biopsies of bony metastases done within 1 week of initial CTC collection in 2 of these patients (patients 6 and 7 in Table 1) revealed no vimentin expression in the

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Table 1. Baseline demographic and clinical characteristics of the men with metastatic CPRC in this study ($n = 41$)

Demographics	$n = 41$
Median age, y (range)	71 (50–89)
Race, ethnicity	
White, non-Hispanic	73%
Black, non-Hispanic	27%
Baseline disease history	
Median Gleason score (range)	8 (5–10)
Median baseline PSA (ng/dL, range)	248.9 (14.0–13,419.5)
Median baseline pain (range)[a]	0 (0–7)
Median Karnofsky performance status (range)	90 (60–100)
Median number of prior hormonal therapies (range)	2.5 (0–5)
Prior chemotherapy	68%
Prior bisphosphonates	73%
Sites of metastatic disease	
Visceral (lung + liver)	54%
Lymph node only	0%
Metastatic to bone:	
Metastatic to bone with lymph nodes (no visceral metastases)	24%
Metastatic to bone without lymph nodes (no visceral metastases)	22%

[a]Pain is scored as a linear analog scale (0–10 range).
Abbreviations: CRPC, castration-resistant prostate cancer; PSA, prostate specific antigen.

CK-positive tumor foci, but strong vimentin expression in the surrounding bone stroma, which lacks CK expression (Fig. 2). These same patients had CTCs taken at the same time as the CT-guided tumor biopsy that commonly expressed coexpressed CK and vimentin. These findings are consistent with invasion and metastasis by CTCs that subsequently undergo EMT/MET or exist in a transitional state; an alternative explanation may be that vimentin coexpression may be heterogeneous in metastases, similar to CTC expression.

Among the next cohort of 11 men with mCRPC, we found CTCs coexpressing N-cadherin and CK in 11/11 (100%) patients, and by this criterion 205/244 (84%) of CTCs were identified as coexpressing these markers (Fig. 1D and E, Table 2 and also see Supplementary Table S3, Supplementary Fig. S1 for real-time control cells and Supplementary Fig. S3 for additional examples). The expression of N-cadherin among CTCs varied from undetectable, determined by the real-time negative controls described in Supplementary Table 1, to very strong (Fig. 1D). This variation was observed among CTCs from individual patients as can be seen by examination of 3 CTCs from patient 11 (Fig. 1E). Although we noted heterogenous vimentin expression among patient and control leukocytes, we did not observe N-cadherin expression among patient or control leukocytes.

Among 10 women with metastatic breast cancer, 9 had detectable CTCs and of these, we found evidence of vimentin coexpression in 7 (78%) patients, and 55/88 CTCs overall (63%) coexpressed vimentin (Supplementary Fig. S2, Table 2 and Supplementary Table 4, and controls in Supplementary Fig. S1). Among another 6 women with detectable CTCs and metastatic breast cancer, 4 had evidence of CK and N-cadherin coexpression, and overall 78/95 CTCs (82%) had N-cadherin expression, with significant heterogeneity in expression in a given individual (Table 2, Supplementary Fig. S3 and controls in Supplementary Fig. S1). These data indicate that the majority of CTCs in patients with metastatic breast cancer and mCRPC coexpress epithelial (EpCAM and CK) and mesenchymal (vimentin, N-cadherin) markers, and thus exist in a transitional phenotypic state, similar to that observed in our preclinical models.

Given these findings, we next sought to better characterize the dual expression of the epithelial E-cadherin with the more mesenchymal N-cadherin on circulating CD45-negative–nucleated cells. Western blot analysis of control cells (T47D, BT549, PC-3, and PBMC cells) indicated a lack of cross-reactivity of our E-, N-, or O-cadherin antibodies against their respective antigens (data not shown), providing assurance that these antibodies are able to measure the intended target antigen. In an additional cohort of 6 men with progressive metastatic CRPC, we characterized all CD45-negative cells that were initially captured by using the standard CellSearch method. Given the limited number of proteins that may be evaluated using IF in an individual cell, we examined the presence of both E- and N-cadherin expression on these CD45-negative cells but were not able to additionally measure CK expression and therefore cannot define a CTC as described above. Interestingly, as shown in Table 3, Figure 3, and Supplementary Figure 1, we found clear evidence for 4 distinct subtypes of CD45-negative cells, including those cells that coexpressed both E- and N-cadherin (20%–71% of cells examined in a given subject), cells that expressed E- but not N-cadherin (2%–22% of cells), cells that expressed N- but not E-cadherin (0%–45% of cells), and cells that lacked both cadherin family members (25%–48% of cells). The proportion of CD45-negative cells that expressed N-cadherin was similar to the proportion that expressed E-cadherin (61% vs. 53%), although this varied greatly in individual men (48%–72% vs. 23%–75%, respectively). Finally, we noted that the subtype of E-cadherin–negative/N-cadherin–positive cells was relatively rare among these EpCAM-captured cells except in 2 men (subjects 39 and 40) who had progressive disease on their most recent treatment with the mTOR inhibitor temsirolimus as part of an experimental protocol. Although these analyses are limited to those cells captured based on EpCAM expression, the results indicate that

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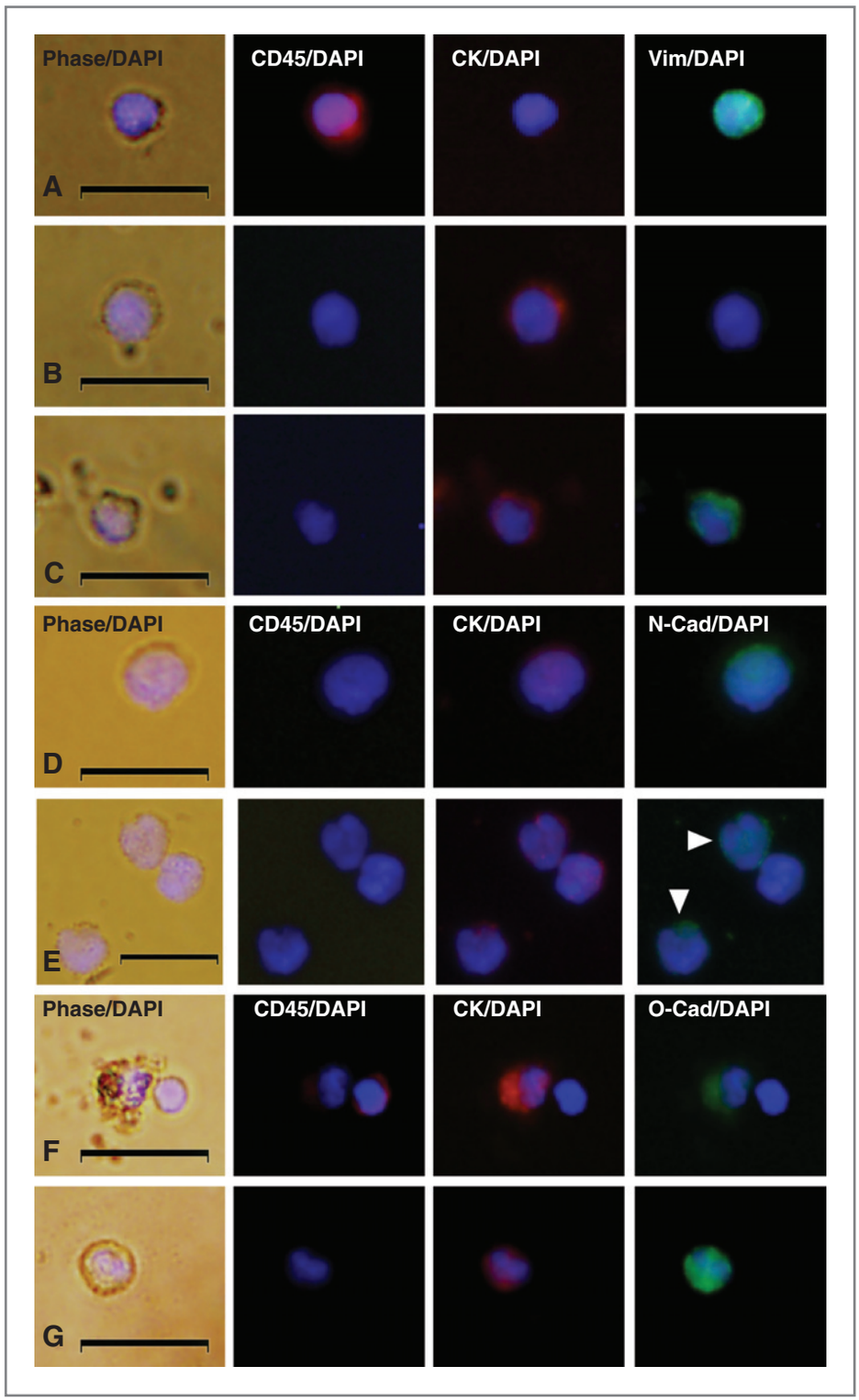

Figure 1. Coexpression of epithelial and mesenchymal proteins in CTCs from men with metastatic castration-resistant prostate cancer (mCRPC). All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI, and either vimentin (Vim)/DAPI, N-cadherin (N-cad)/DAPI expression, or O-cadherin (O-cad)/DAPI as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CTC with no vimentin expression, (C) a CTC with vimentin expression, (D) a CTC with N-cadherin expression, (E) 3 CTCs, 2 with N-cadherin expression (arrowheads), (F) a CTC with O-cadherin expression and a nearby leukocyte, and (G) an additional CTC with O-cadherin expression. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

CTCs coexpress epithelial and mesenchymal proteins and suggest the existence of currently undetected CTCs with reduced or absent epithelial markers. Methods that evaluate whole blood for non-leukocyte, nucleated cellular populations without initial EpCAM-based capture may thus be able to identify these additional cells.

Given that prostate cancer has a tendency to metastasize to bone, we next hypothesized that adhesion molecules that

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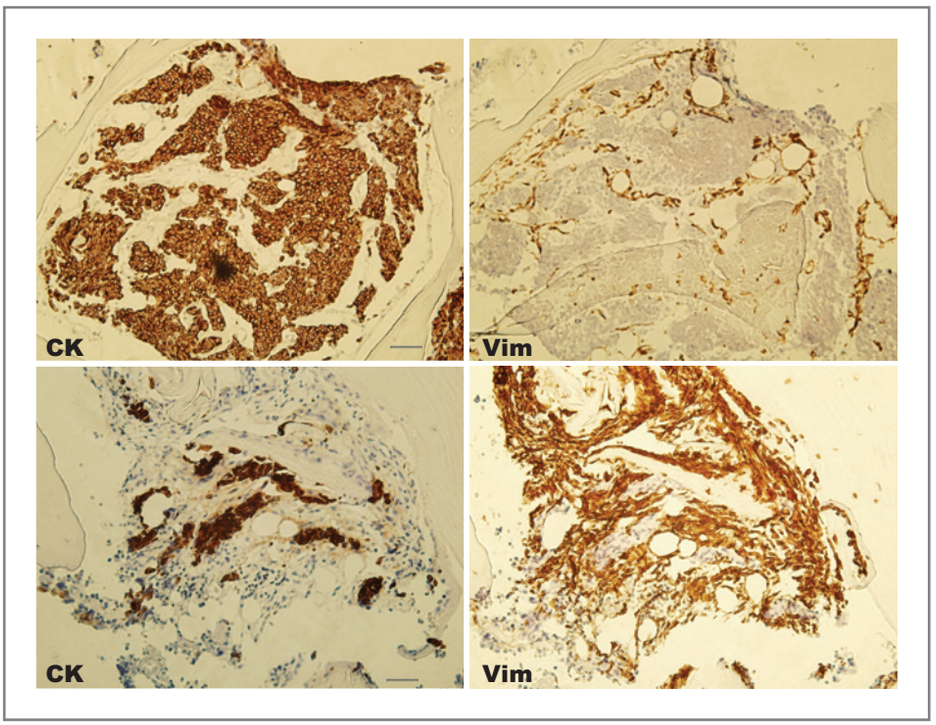

Figure 2. Expression of vimentin and CK in prostate cancer metastases. Images are taken from a CT-guided targeted bone metastasis biopsy at the same time as CTCs were collected and evaluated for vimentin coexpression by immunofluorescence as described. Images are from patient 6 (top) and patient 7 (bottom), with CK (left), and vimentin (right) expression assayed by IHC (20× magnification). Scale bars in the CK panels represent 50 μm and were added from an image taken at identical magnification and resolution.

favor an osteoblastic tumor microenvironment may be visualized on CTCs from men with CRPC. O-cadherin has been recently linked to metastasis to bone in preclinical models of prostate cancer (34, 35), and we thus sought to examine its coexpression with CK in CTCs. Indeed, we found coexpression in 6 of 6 (100%) of men with CRPC and detectable CTCs by EpCAM-based ferromagnetic capture, and O-cadherin was expressed in 64%–96% of detectable CTCs (e.g., shown in Fig. 1F and G, summarized in Table 2, and Supplementary Table S3 with controls in Supplementary Fig. S1). These findings suggest that prostate cancer CTCs coexpress adhesion molecules that may promote homing to bone and homotypic binding to osteoblastic cells.

Given the expression of the stem cell–associated antigen CD133 in putative prostate cancer stem cells and other cancer stem cell populations (27, 33, 36), we investigated CD133 expression in CTCs from men with mCRPC. We found CD133 to be expressed in 11/11 (100%) men with CTCs, and in 127/153 (83%) of CTCs from these men (Fig. 4, Table 2, Supplementary Fig. S4 and controls in Supplementary Fig. 1). These data suggest that CTCs from patients with common epithelial malignancies coexpress both epithelial and mesenchymal markers, suggesting that EMT/MET transitions may be contributing to metastatic progression. In addition, in men with metastatic CRPC, the coexpression of the stemness antigen CD133 in the majority of CTCs suggests that these cells may have acquired properties of stemness during their migration into the bloodstream (21, 24).

Discussion

In these studies, we have identified biomarkers suggestive of epithelial plasticity and stemness in CTCs from patients with common metastatic epithelial malignancies, including breast and prostate cancer. The identification of both epithelial and mesenchymal phenotypes among CTCs in a significant subset of patient samples offers several important clinical opportunities. These data suggest that CTCs may undergo phenotypic changes from epithelial to more mesenchymal transitional states during metastatic transit, whereas metastases themselves may be more epithelial in phenotype and marker expression. Our findings also suggest that in addition to cells expressing both epithelial and mesenchymal markers, there may be an unknown number of CTCs that are more mesenchymal-like and thus are EpCAM negative. These cells will be missed by the FDA-approved CellSearch method, the Adna Test (AdnaGen AG) system, and current microfluidic technologies, which enrich for CTCs by immunoabsorbtion of cells expressing MUC1 or EpCAM (37). Indeed, recent studies in breast cancer have suggested that "normal" type breast cancer cell lines that overexpress EMT and stem cell antigens ($CD44^+$, $CD24^-$) may lack EpCAM and are thus not detectable by currently approved CTC detection systems (38). Therefore, it is possible that the number of CTCs in patients with metastatic cancer is much higher than currently appreciated.

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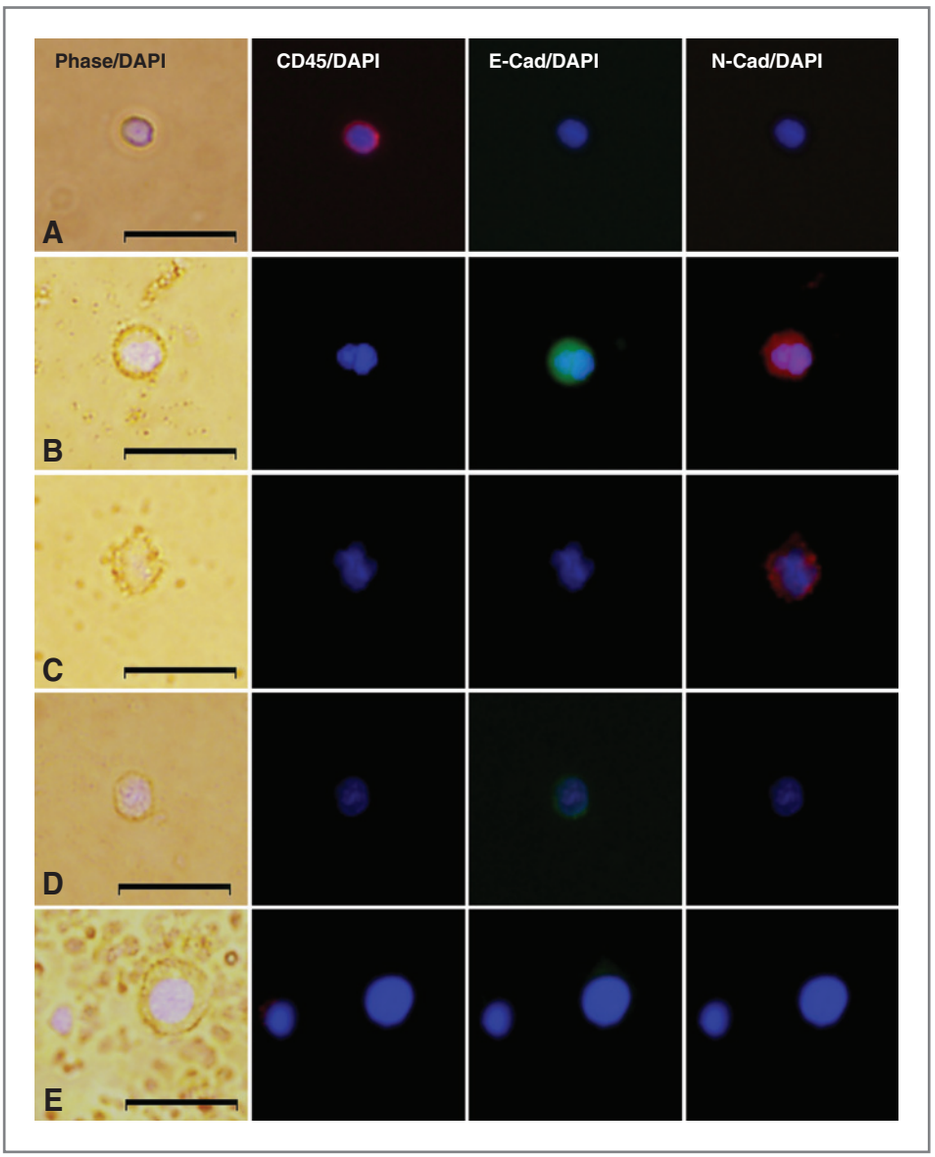

Figure 3. E-cadherin and N-cadherin coexpression among CD45-negative–nucleated cells from men with metastatic CRPC. All panels represent merged immunofluorescent images derived from phase/DAPI, CD45/DAPI, E-cadherin (E-cad)/DAPI, and N-cadherin (N-cad)/DAPI expression as indicated. A, a leukocyte with CD45 expression, (B) a CD45-negative–nucleated cell, with E-cadherin and N-cadherin coexpression, (C) a CD45-negative–nucleated cell with N-cadherin expression and no E-cadherin expression, (D) a CD45-negative–nucleated cell with E-cadherin expression and no N-cadherin expression, and (E) a nucleated cell lacking expression of CD45, E-cadherin, and N-cadherin. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

It is well appreciated that cells induced to undergo EMT activate stem cell pathways (24). Indeed, a recent study showed a striking relationship between prostate cancer–associated fibroblast chemokine expression of interleukin-6 or TGF-β and the acquisition of tumor invasiveness, metastatic propensity, EMT antigen expression, and stemness characteristics (39). Our findings suggest that CTCs captured by using an epithelial-based capture assay, the current FDA standard method, express the stemness marker CD133. These findings are consistent with the results of Mani and colleagues (24) showing a relationship between EMT and stemness states in breast cancer models. It is tempting to speculate that these CTCs may represent transitional cells with both epithelial and mesenchymal phenotypes, and this heterogeneity or plasticity may also extend to stem cell markers and to treatment-induced effects. For example, we observed an increased predominance of N-cadherin expression in CTCs from 2 men who were progressing on their most recent treatment with an mTOR inhibitor, suggesting that certain systemic agents may alter CTC phenotype. An alternative explanation to this transitional state theory is that tumor heterogeneity for these markers exist, without the need to transition phenotypically. Further preclinical and clinical studies of metastatic tumors in which the EMT/MET process is experimentally disrupted or induced may shed further light on these observations.

Our working model of plasticity also predicts that cells with maximal stem cell character, which by definition will be highly malignant, should display both epithelial and mesenchymal traits, because they inhabit intermediate states in the epithelial-mesenchymal axis. Here, we show that

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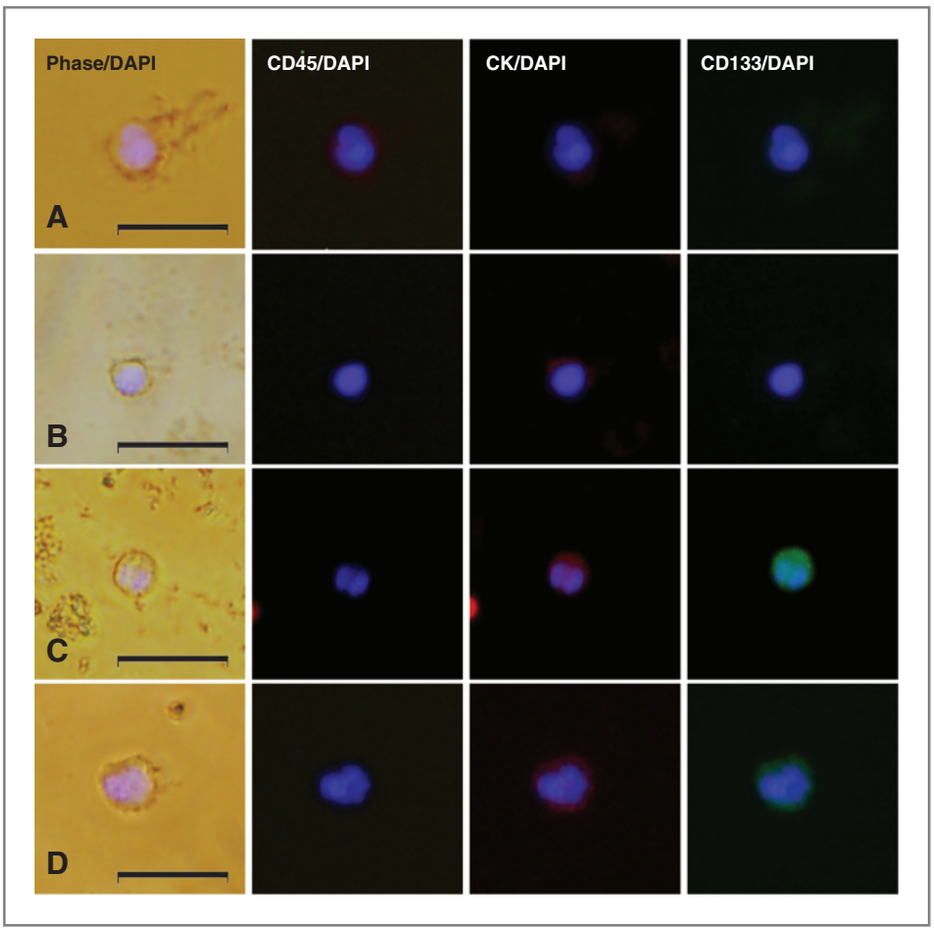

Figure 4. Expression of the stem-like cell marker CD133 by immunofluorescence in CTCs from men with mCRPC. All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI, and CD133/DAPI expression as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CD133-negative CTC, (C) a CD133-positive CTC, and (D) an additional example of a CD133-positive CTC. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

patients with metastatic breast and prostate carcinomas have CTCs that exist in phenotypic states that may be intermediate to epithelial and mesenchymal states. Although the enumeration of EpCAM-captured CTCs or CD133-positive CTCs correlates with disease progression, there is great heterogeneity to the number of CTCs isolated from individual patients with metastatic carcinomas, and thus a further refinement in the methods of CTC detection by using EMT

Table 2. Prevalence of EMT and stemness antigen marker expression by immunofluorescence in CTCs (intact, nucleated CK^+, $CD45^-$, DAPI + cells) from the circulation of men with metastatic CRPC and women with metastatic breast cancer

Antigen	*n*	Marker-positive CTCs (%)	Patients with marker-positive CTCs (%)
Vimentin (CRPC)	10	108/126 (86%)	10/10 (100%)
Vimentin (BC)	10	65/97 (65%)	7/10 (70%)
N-Cadherin (CRPC)	11	205/244 (84%)	11/11 (100%)
N-Cadherin (BC)	6	78/95 (82%)	4/6 (67%)
O-Cadherin (CRPC)	6	107/120 (89%)	6/6 (100%)
CD133 (CRPC)	11	127/153 (83%)	9/11 (82%)

NOTE: Columns on the right indicate the number of manually scored CTCs scoring positive for each marker and the number of patients in each group who have at least 1 CTC that stains positive for a given marker.
Abbreviation: CRPC, castration-resistant prostate cancer; BC, breast cancer.

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Table 3. Prevalence of E-cadherin and N-cadherin expression on CD45-negative–nucleated cells isolated from the circulation using an EpCAM-based ferrofluid (CellSearch method) among men with progressive metastatic CRPC

Subject number	Clinical CTC	CD45-negative cells	E + N + cells (%)	E − N − cells (%)	E + N − cells (%)	E − N + cells (%)
35	7	24	17 (71%)	6 (25%)	1 (4%)	0 (0%)
36	55	23	11 (48%)	8 (35%)	4 (17%)	0 (0%)
37	112	122	82 (67%)	30 (25%)	4 (3%)	6 (5%)
38	1000	65	27 (42%)	22 (34%)	14 (22%)	2 (3%)
39[a]	45	44	9 (30%)	21 (48%)	1 (2%)	13 (30%)
40[a]	16	83	18 (22%)	26 (31%)	2 (22%)	37 (45%)
Summary	1235	361	164 (45%)	113 (31%)	26 (7%)	58 (16%)

NOTE: Clinical CTC = enumeration by using the FDA-approved method. Each column represents the number and percentage of CD45-nucleated cells that were identified based on dual marker expression.

[a]Indicates 2 patients with metastatic CRPC who were progressing on therapy with the mTOR inhibitor temsirolimus.

antigen–based capture methods may result in improved prognostication CTC identification (27–29). Aktas and colleagues recently showed that a population of cells enriched in CTCs expressed RNAs encoding mesenchymal markers; however, this study could not prove coexpression of epithelial and mesenchymal markers in the same cell (40). On the basis of the results presented here, further studies to explore methods to capture cells based on these markers in addition to EpCAM are warranted to investigate their relevance to metastatic progression and chemoresistance.

There are several inherent limitations to this work. First, we have not shown true stemness among CTCs, which would require further experimental evidence of serial clonal passage and transplantation, or prolonged culture of CTCs from patients. This is not technically feasible with current methods. Our markers of stemness are correlative in nature only, and may be associated with properties other than stemness. We also acknowledge that coexpression of epithelial and mesenchymal markers does not, in itself, represent plasticity, as we are unable to observe this dynamic *in vivo* in patients. Our clinical observations suggest plasticity based on the coexpression in real time on CTCs during the process of metastasis, and the lack of expression of vimentin in paired metastases from the same patients. The importance of this plasticity to highly aggressive metastatic behavior can only be tested through experimental manipulation of preclinical systems in which either EMT/MET is prevented; future experiments will need to address this issue. Finally, these studies have not correlated coexpression of EMT factors on CTCs with clinical outcomes; these prognostic studies require large appropriately powered studies and patients with long-term follow-up, such as has been recently reported with CD133-positive colorectal CTCs and postoperative outcomes (27). Our findings, however, suggest that the measurement of CTCs collected through both EpCAM-enriched and EMT antigen–enriched methods may complement each other in providing prognostic or predictive information during systemic therapy that should be prospectively evaluated.

Finally, CTCs expressing mesenchymal or stem like markers expression, which comprise the majority of cells isolated in this study, and additional cells that may go undetected due to EpCAM loss, represent a therapeutic problem. It has been well documented that EMT alters drug sensitivity (21, 41, 42) and it has been challenging to direct therapy to cancer cells with stem cell–like properties, perhaps because of their recalcitrance to undergo apoptosis (43). Although recent studies suggest both a screening method and actual compounds (e.g., salinomycin) that can selectively target cancer stem cells (21), these aggressive cells still represent a formidable challenge. Our findings suggest that these cell types may be highly prevalent among patients with metastatic epithelial tumors, and suggest methods for the improved detection of these cells *in vivo* to assist in developing novel therapeutic strategies.

Disclosure of Potential Conflicts of Interest

A.J. Armstrong, S. Oltean, D. George, and M.A. Garcia-Blanco are listed as inventors in a related patent application (application number PCT/US10/50233) filed on September 24, 2010.

Authors' Contributions

A.J. Armstrong, S. Oltean, D. George, and M.A. Garcia-Blanco conceived of the original study. A.J. Armstrong and M.A. Garcia-Blanco directed the research. G. Kemeny, S. Oltean, R. Bitting, and M.S. Marengo carried out laboratory experiments. A.J. Armstrong, J. Turnbull, C.I. Herold, P.K. Marcom, and D. George carried out clinical procedures and recruitment. Although all coauthors contributed to the writing of the manuscript, A.J. Armstrong, M.S. Marengo, and M.A. Garcia-Blanco did the majority of writing and editing.

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Supplementary Data

Antigen	Product	Positive Control	Negative Control	Leukocyte Expression	Dilution
Vimentin (IF)	BD Biosciences, mouse monoclonal IgG1, 550513	PBMCs, PC-3, DU145	T47D, LnCAP, mock	Yes	2:225
N-cadherin (IF, WB)	BD Biosciences (San Jose, CA), mouse monoclonal IgG1, 610920	Sarcoma, rat brain, PC-3	PBMCs, mock	No	4:225
Cytokeratin (pan-CK, IF)	AbD Serotec (Raleigh, NC), mouse monoclonal IgG1, MCA1907HT	T47D, DU145	PBMCs, mock	No	10:225
CD45 (IF)	Invitrogen (Carlsbad, CA), mouse IgG1, HI30, MHCD4500	PBMC	PC-3, DU145, mock	Yes	5:225
CD133 (IF) (PROM1)	Novus Biologics (Littleton, CO), polyclonal rabbit, NB120-16518	CaCo-2 colon cancer cells	PMBCs, mock	Variable	4:225
O-cadherin (IF, WB)	Invitrogen (Carlsbad, CA), mouse IgG1 kappa, 5B2H5, 32-1700	PC-3	PBMCs, mock, T47D	No	5:225
E-cadherin (IF, WB)	BD Biosciences (San Jose, CA), mouse IgG2a, 612131	T47D, PC-3	PMBCs, mock	No	4:225

Supplementary table 1. EMT/stemness antigens assessed in CTCs and control cells. IF=immunofluorescence. WB=western blot.

DEMOGRAPHICS	***N=16***
Median Age (range)	61 (48-81)
Race, Ethnicity	
White, non-hispanic	44 %
Black, non-hispanic	50 %
Asian, non-hispanic	6 %
BASELINE DISEASE HISTORY	
ER/PR positivity	56% / 50%
HER (negative, 1+, 2+)	60% , 27%, 13
Median Baseline Pain, range	0 (0-6)
Median Karnofsky Performance Status (range)	90 (70-90)
Median # of Prior Hormonal Therapies (range)	1 (0-4)
Prior Chemotherapy	81%
SITES OF METASTATIC DISEASE	
Visceral (lung or liver)	75%
Lymph node only	0 %
Lymph Node/, soft tissue, or contralateral breast only	13%
Bone Metastatic:	
Bone metastases with lymph nodes (no visceral metastases)	0 %
Bone metastases without lymph nodes (no visceral metastases)	13 %

Supplementary Table 2. Baseline demographic and clinical characteristics of the women with metastatic BC in this study (n=16). Pain is scored as a linear analog scale (0-10 range).

Subject Number	CTC Count (Cellsearch)[i]	Vimentin (+) CTCs / Total Manual CTC Count[ii]
1	5	4/6
2	4	2/2
3	54	11/11
4	45	6/10
5	626	5/8
6	110	17/21
7	182	5/6
8	17	13/16
9	19	33/34
10	34	12/12
Total	**1127**	**108/126 (86%)**
Subject Number	**CTC Count (Cellsearch)**	**N-Cadherin (+) CTCs/ Total Manual CTC Count**
11	45	13/19
12	12	5/7
13	10	8/8
14	5	7/8
15	12	3/4
16	220	11/13
17	828	81/96
18	26	6/11
19	12	18/22
20	42	15/18
21	485	38/38
Total	**1697**	**205/244 (84%)**
Subject Number	**CTC Count (Cellsearch)**	**CD133 (+) CTCs/ Total Manual CTC Count**
22	16	6/11
23	91	15/21
24	6	0/0
25	36	29/29
26	27	9/9
27	43	10/15
28	2	0/0
29	23	12/14
30	38	23/26
31	30	12/17
32	75	11/11
Total	**387**	**127/153 (83%)**
Subject Number	**CTC Count (Cellsearch)**	**O-Cadherin (+) CTCs/ Total Manual CTC Count**
33	198	23/24
34	180	18/20
35	102	9/14
36	7	15/15
37	55	11/14
38	112	31/33
Total	**654**	**107/120 (89%)**

Supplementary Table 3. Prevalence of EMT and stemness marker expression in individual subjects with metastatic CRPC from this study.

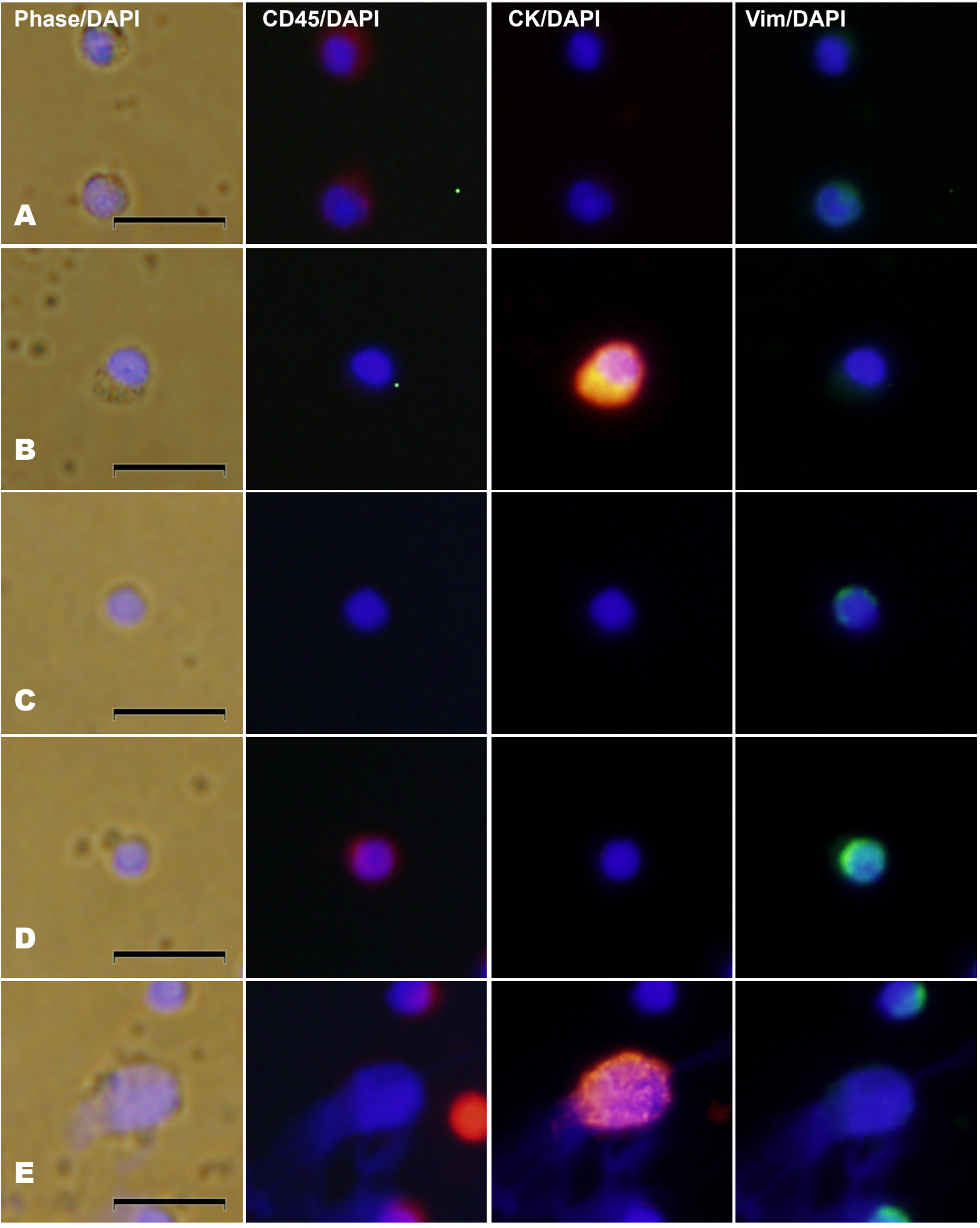
[i] The middle column represents the CTC Count from the FDA-approved Cellsearch® enumeration of CTCs for each subject.

[ii] Right column represents the ratio of vimentin (co-expression of vimentin ranged from 60-100% of cells in a given individual), N-cadherin (Co-expression of N-cadherin ranged from 55-100% of cells in a given individual), CD133 (CD133 co-expression ranged from 55-100% of evaluable cells in a given individual), or O-cadherin (Co-expression of O-cadherin ranged from 64-100% of evaluable cells in a given individual) expressing CTCs among the total number of CTCs that were manually enumerated. A CTC was defined as an intact DAPI positive (nucleated) cell that lacked CD45 expression and expressed cytokeratin.

Subject Number	CTC Count (Cellsearch)	Vimentin (+) CTCs / Total Manual CTC Count
1	21	0/6
2	7	2/2
3	8	4/4
4	21	1/2
5	12	2/2
6	188	21/22
7	324	29/33
8	377	6/23
9	0	0/0
10	3	0/3
Total	**961**	**65/97 (67%)**
Subject Number	**CTC Count (Cellsearch)**	**N-Cadherin (+) CTCs / Total Manual CTC Count**
11	1062	9/13
12	2	0/3
13	147	52/59
14	6	2/5
15	33	15/15
16	2	0/0
Total	**1252**	**78/95 (82%)**

Supplementary Table 4. Expression of vimentin and N-cadherin on CTCs from serial cohorts of 16 women with metastatic progressive breast cancer. The second column is the enumerated CTC count using the approved CellSearch® method, while the third column represents the ratio of EMT marker positive CTCs manually scored compared with the total number of CTCs manually scored from each sample.

Supplementary Data: Figures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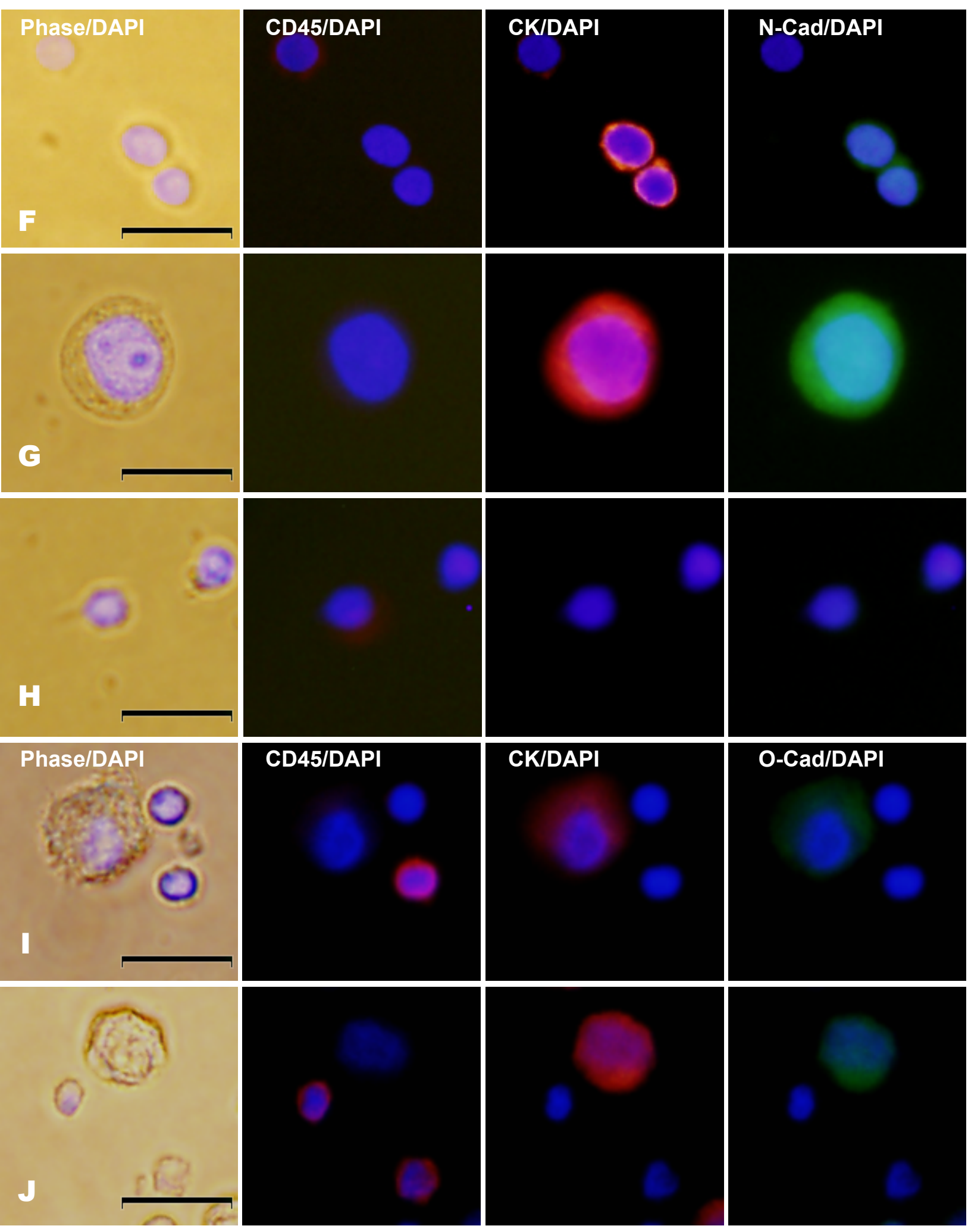
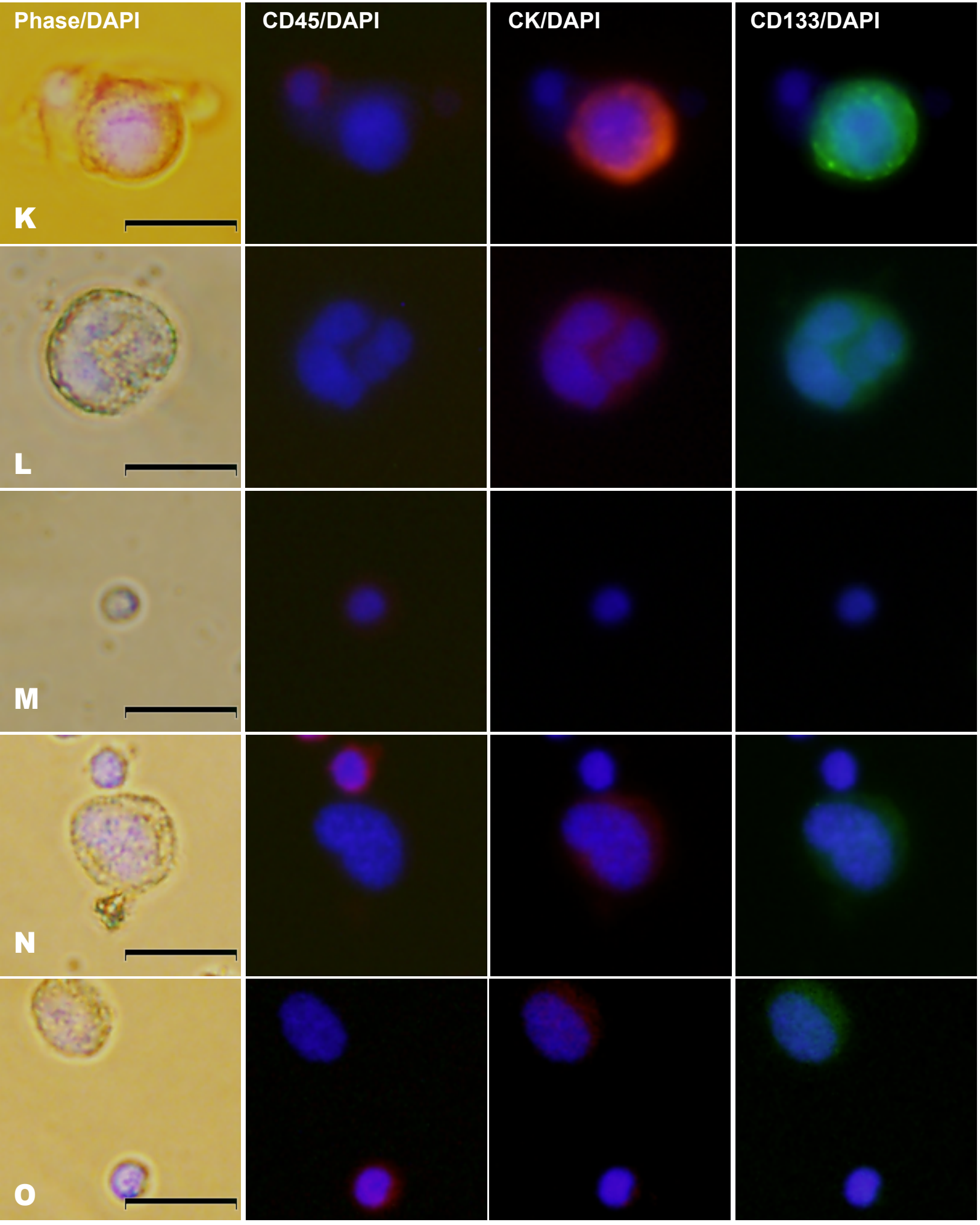
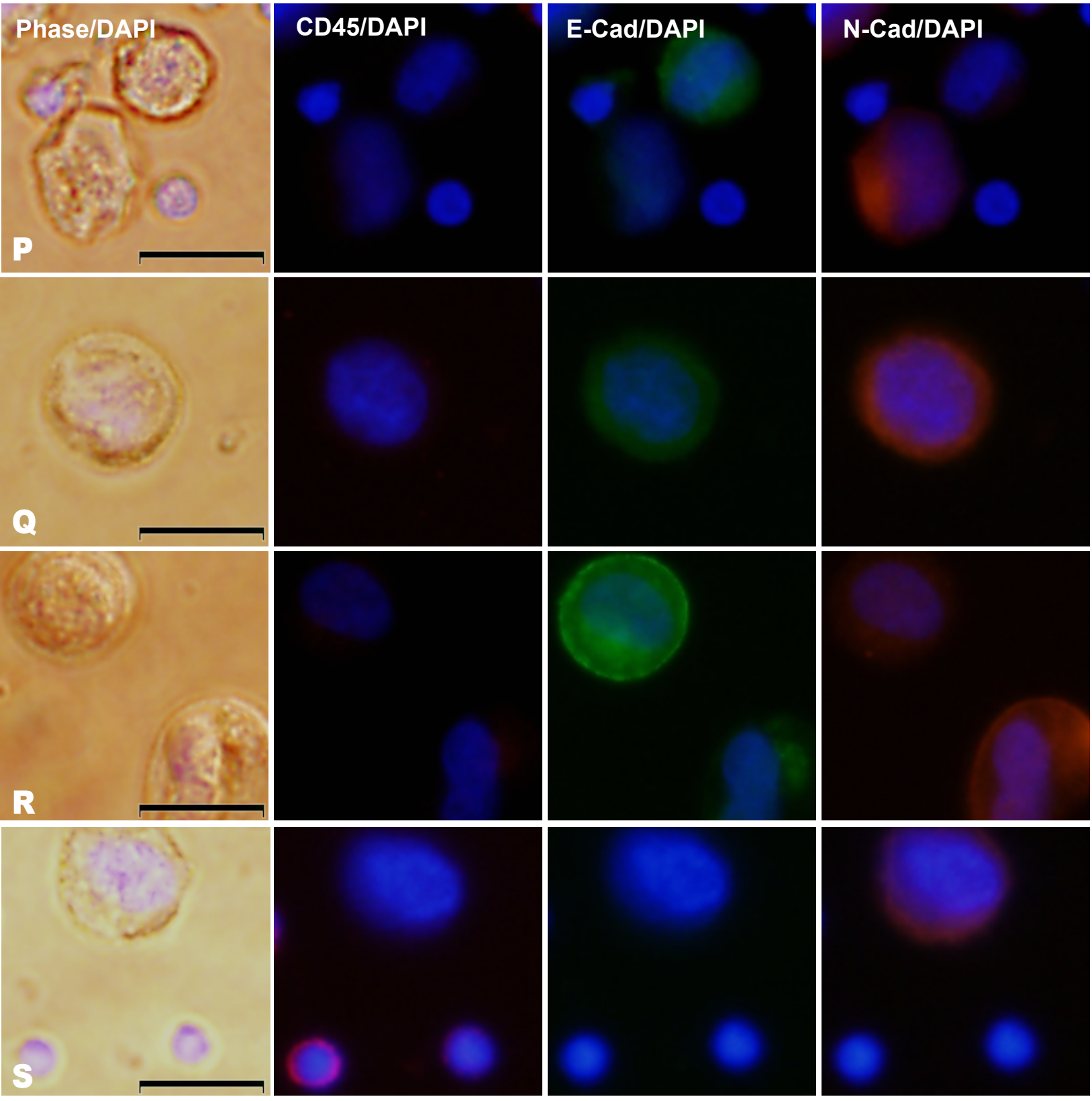
CD45/DAPI
CK/DAPI
Vim/DAPI
A
B
C
D
E


Phase/DAPI
CD45/DAPI
CK/DAPI
N-Cad/DAPI
F
G
H
Phase/DAPI
CD45/DAPI
CK/DAPI
O-Cad/DAPI
I
J


Phase/DAPI
CD45/DAPI
CK/DAPI
CD133/DAPI
K
L
M
N
O

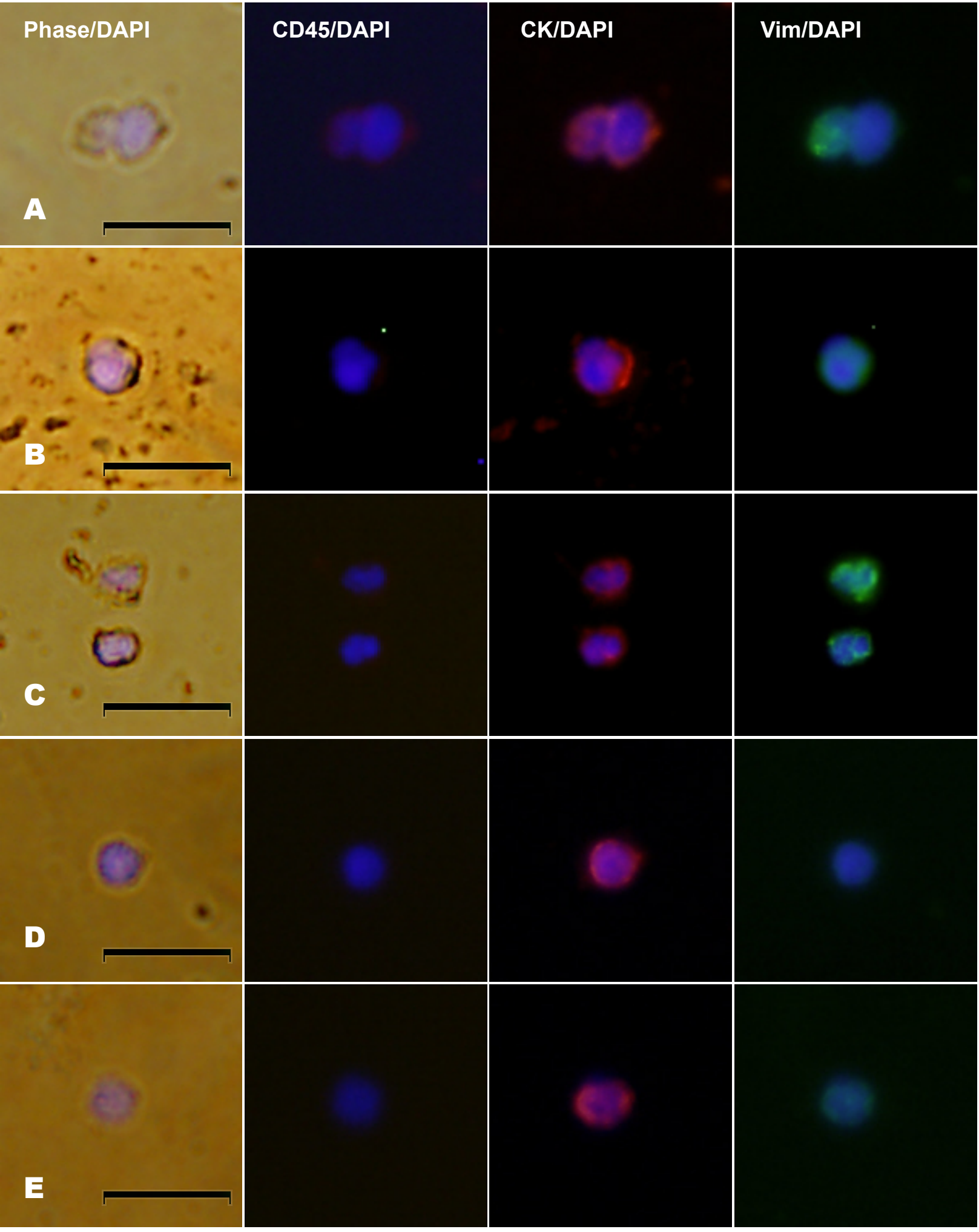


Supplementary Figure 1

Supplementary Figure 1. Mixtures of control cells were assayed in parallel with the patient samples. Scale bars represent 20 μm and were added from a cell image taken from identical magnification and resolution. (A) Parallel control for Fig. 1A: cells that express CD45, lack CK expression, and have variable vimentin expression. (B) Parallel control for Fig. 1A: cell that lacks CD45 expression, expresses CK and has low expression of vimentin. (C) Parallel control for Fig. 1B and 1C (same patient sample, same day of assay): cell that lacks CD45 and cytokeratin expression and expresses vimentin. (D) Parallel control for Fig. 1B and 1C: cell that expresses CD45 and vimentin but lacks CK expression. (E) Parallel control for Fig. 1B and 1C: cell that lacks CD45 expression, expresses CK, and lacks vimentin expression. Note reciprocal expression in nearby cells. (F) Parallel control for Fig. 1D: cell that expresses CD45 and lacks CK and N-cadherin expression. Nearby, two cells that lack CD45 expression and express CK and N-cadherin. (G) Parallel control for Fig. 1E: cell that lacks expression of CD45 and expresses CK and N-cadherin. (H) Parallel control for Fig. 1E: cell that expresses CD45 and lacks CK and N-cadherin expression. (I) Parallel control for Fig. 1F: one large cell that lacks expression of CD45 and expresses CK and O-cadherin. Note nearby cell with CD45 expression and another nearby cell with no expression of CD45, CK, or O-cadherin. (J) Parallel control for Fig. 1G: two cells with CD45 expression and one cell lacking CD45 and expressing CK and O-cadherin.

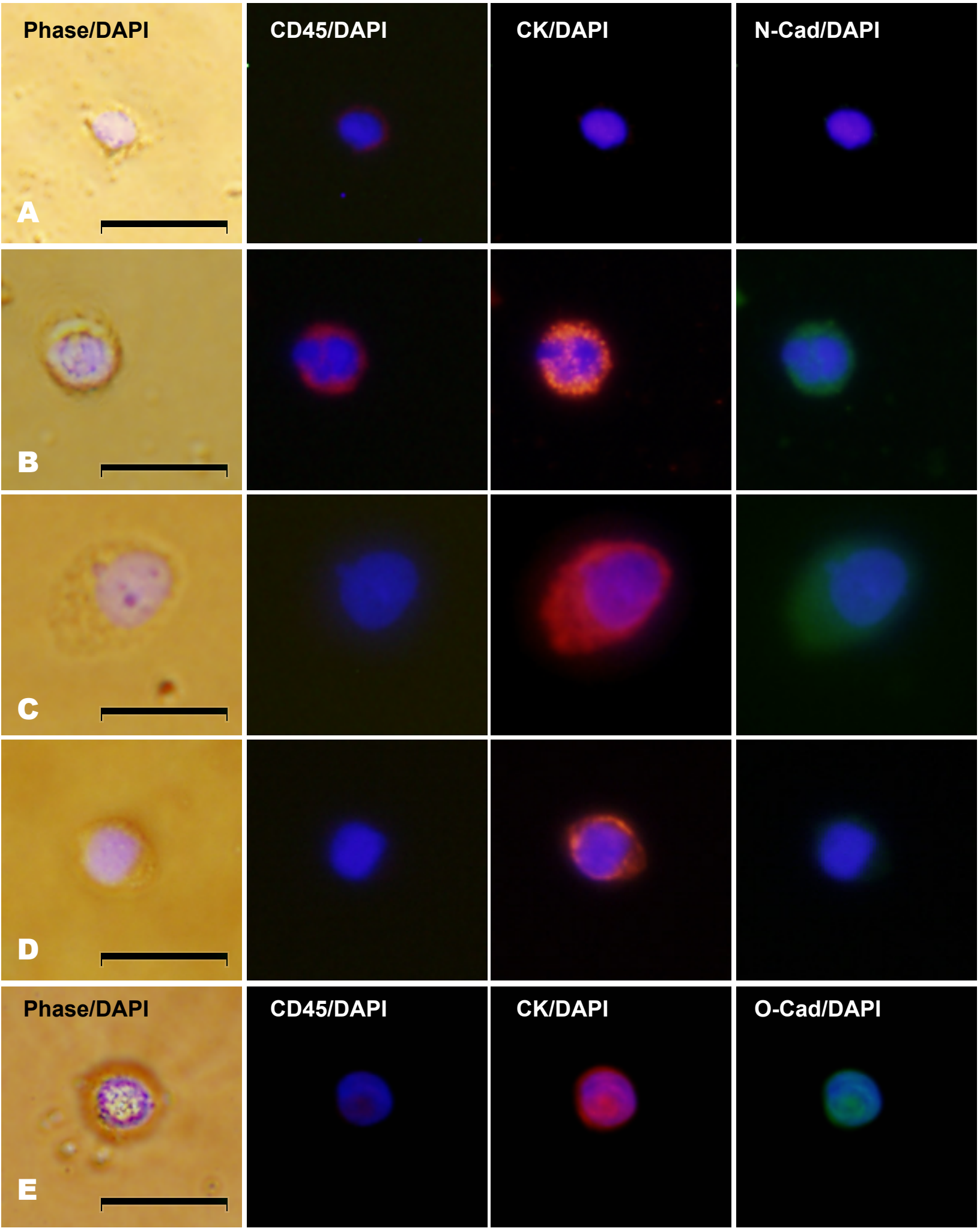
(K) Parallel control for Fig. 3A: cell that expresses CD45 and lacks CK and CD133 expression. Nearby, a cell that lacks CD45 expression and expresses CK and CD133. (L) Parallel control for Fig. 3B: cell that lacks CD45 expression and expresses CK and CD133. (M). Parallel control for Fig. 3B: cell that expresses CD45 and lacks CK and CD133 expression. (N) Parallel control for Fig. 3C: cell that expresses CD45 and lacks CK and CD133 expression. A nearby cell lacks CD45 expression and expresses CK and CD133. (O) Parallel control for Fig. 3D: cell that expresses CD45 and lacks CK and CD133 expression. A nearby cell lacks CD45 expression and expresses CK and CD133.

(P) Parallel control for Fig. 4A: one cell with only E-cadherin expression and another cell with only N-cadherin expression. (Q) Parallel control for Fig. 4B and 4C (same patient sample, same day of assay): cell with expression for both E-cadherin and N-cadherin. (R) Parallel control for Fig. 4D: one cell with strong E-cadherin expression and weak N-cadherin expression and a second cell with weak E-cadherin expression and strong N-cadherin expression. (S) Parallel control for Fig. 4E: one cell with only N-cadherin expression and two cells with only CD45 expression.



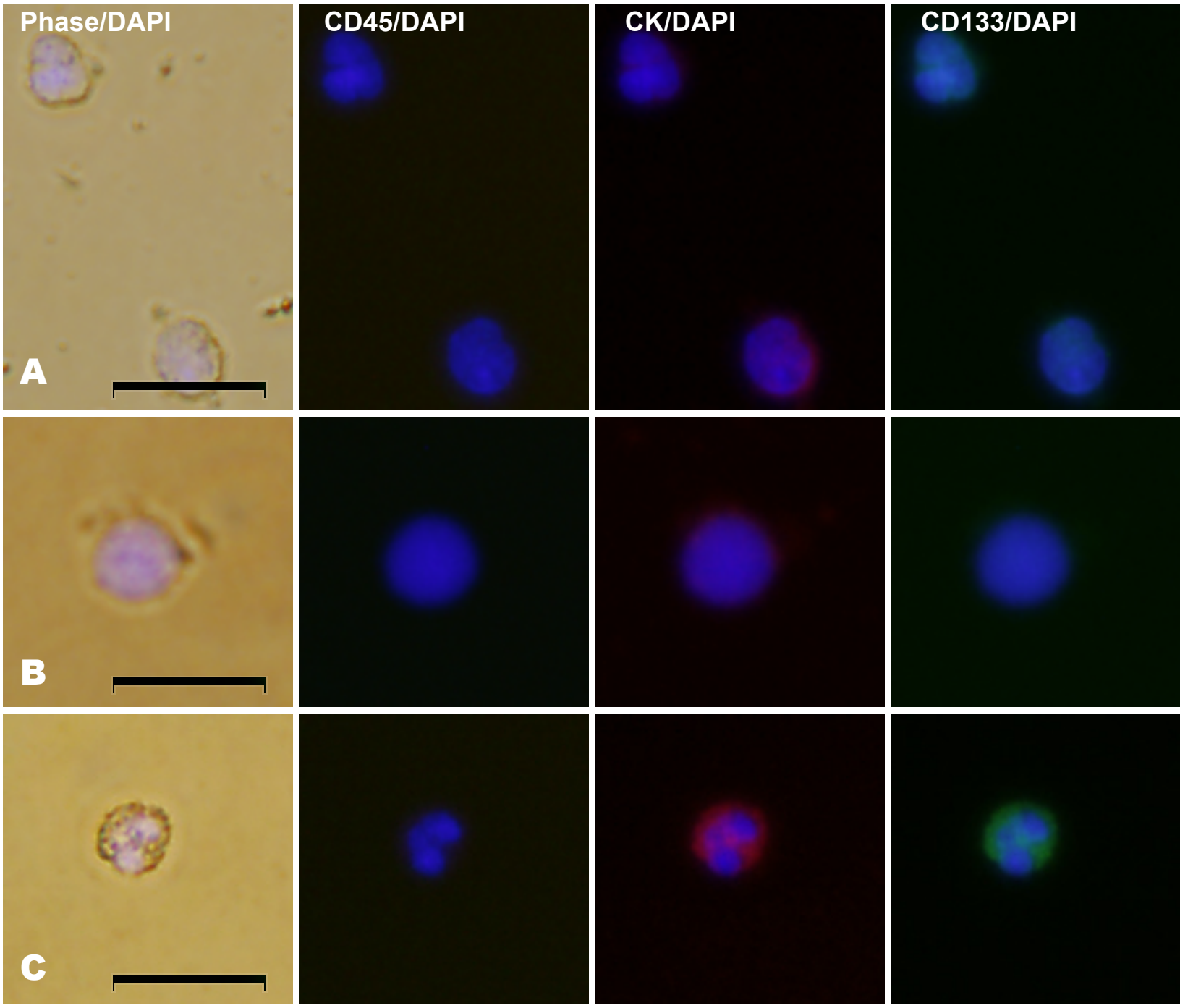
Supplementary Figure 2

Supplementary Figure 2. Additional examples of CTCs based on vimentin expression. Vimentin expression in CTCs from men with progressive metastatic CRPC, (A-C) or women with progressive metastatic breast cancer (D,E). Columns indicate phase/DAPI, CD45/DAPI, CK/DAPI, and vimentin/DAPI expression as indicated. (A) Two CTCs, one with (left) and one without (right) vimentin expression. (B,C) Further examples of vimentin positive CTCs. (D) A CTC from a woman with mBC that lacks vimentin expression. (E) A CTC from a woman with mBC that expresses vimentin.



Supplementary Figure 3

Supplementary Figure 3. Additional examples of CTCs based on N-cadherin or O-cadherin expression. CTCs from men with progressive metastatic CRPC (A, B, E) and women with progressive mBC (C,D). Scale bars represent 20 μm and were added from a cell image taken from identical magnification and resolution. Columns indicate phase/DAPI, CD45/DAPI, CK/DAPI, and N-cadherin/DAPI or O-cadherin expression as indicated. (A) A patient leukocyte with negative N-cadherin expression. (B) A cell of unknown character that expresses CD45, N-cadherin, and cytokeratin (triple positive cell). (C) A CTC from a woman with mBC that expresses N-cadherin. (D) A CTC from a woman with mBC that lacks N-cadherin expression. (E) A CTC from a man with CPRC with CK and O-cadherin expression and no CD45 expression.



Supplementary Figure 4

Supplementary Figure 4. Additional CTCs based on CD133 expression. CD133 expression on CTCs from men with progressive metastatic CRPC, (A-C). Scale bars represent 20 μm and were added from a cell image taken from identical magnification and resolution. Columns indicate phase/DAPI, CD45/DAPI, CK/DAPI, and N-cadherin/DAPI expression as indicated. (A) Two cells with variable CK and CD133 expression. (B) A CTC that lacks CD133 expression. (C) A CTC with strong CD133 expression. Because of its nuclear morphology this cell resembled a leukocyte, but given the criteria defined above (CD45 negative and CK positive) it was scored as a CTC.